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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Christian P. Larsen et al.

Serial No.: 10/057,288 Examiner: P. Gambel, Ph.D.

Filed: January 25, 2002 Group Art Unit: 1644

Title: METHODS OF INDUCING ORGAN TRANSPLANT TOLERANCE AND CORRECTING HEMOGLOBINOPATHIES USING L104EA29YIg IN CONJUNCTION WITH AN ALKYLATING AGENT

572 East Green Street, Suite 203
Pasadena, CA 91101
December 26, 2007

MAIL STOP APPEAL BRIEF - PATENTS
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

APPEAL BRIEF UNDER 37 C.F.R. §41.37 AND PETITION FOR THREE MONTH EXTENSION OF TIME UNDER 37 C.F.R. §1.136

Applicants hereby submit an Appeal Brief with a request for a three month extension of time to the Board of Patent Appeals and Interferences ("the Board") of the United States Patent and Trademark Office ("Office") pursuant to 37 C.F.R. §§41.37 and 1.136. Applicants filed a Pre-appeal Brief Request to the final Office Action concurrently with a Notice of Appeal on July 26, 2007. On August 30, 2007, after reviewing applicants' Pre-appeal Brief Request, the Office maintained the outstanding rejections. Applicants hereby petition for a three month extension of time. The fee for a three month extension of time for large entity is one thousand and fifty dollars ($1050.00). Applicants enclose a check including the amount of $1050.00 to cover the extension fees. An Appeal Brief with a three month extension of time is now due December 26, 2007. Accordingly, this Appeal Brief is being timely filed.

A Request for Oral Hearing is attached as Exhibit A.

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02 FC:1402 510.00 OP

Fees totaling $2590.00 are now due for an Appeal Brief in support of an appeal ($1050.00 for a three month extension fee under CFR 17(a)(3); $510.00 under 37 C.F.R. §41.20(a)(3) for filing an Appeal Brief; and $1030.00 for Request for Oral Hearing under 37 C.F.R. §41.20(b)(3)). A check in the amount of $2590.00 is enclosed. Accordingly, this Appeal Brief and the Request for Oral Hearing are being timely filed.

A. TABLE OF CONTENTS

B. REAL PARTY IN INTEREST

The parties named in the caption are the inventors of the claimed methods. Further, the inventors have assigned their entire interest in the subject application to Emory University and Emory has licensed its interest in the subject application to Bristol-Myers Squibb Company.

The real parties of interest are Emory University and Bristol-Myers Squibb Company.

C. RELATED APPEALS AND INTERFERENCES

At the present time, there are no pending appeals or interferences related to this case.

D. STATUS OF CLAIMS

As of the Office Decision dated August 30, 2007:

Claims 1, 2, 5, 6, 9, 10, 12, 13, 30, 33, 34, 36, 37, 44-52, 54-60, and 62-74 were pending and rejected by the Patent Office.

Claims 3, 4, 7, 8, 11, 14-29, 31, 32, 35, 38-43, 53 and 61 have been cancelled.

The rejection of Claims 1, 2, 5, 6, 9, 10, 12, 13, 30, 33, 34, 36, 37, 44-52, 54-60, and 62-74 is being appealed.

E. STATUS OF AMENDMENTS

The final Office Action dated March 29, 2007, entered the Applicants' Amendment filed on December 13, 2006.

No amendments were filed after issuance of the final Office Action dated March 29, 2007.

F. SUMMARY OF THE CLAIMED SUBJECT MATTER

Applicants' invention (as embodied in independent Claims 1, 9, 34, 55, 56, 62 and 63) is directed to methods involving generally the heretofore unknown discovery that rejection of a solid organ or tissue/cellular transplant in a subject can be inhibited or reduced by the following sequence of steps:

- -- administering T cell depleted bone marrow cells to the subject before, during or after the solid organ or tissue/cellular transplant;
- -- administering an alkylating agent (e.g., busulfan) to the subject in an amount that facilitates mixed chimerism;
- -- administering a subsequent dose of T cell depleted bone marrow; and
- -- administering an immunosuppressive composition that blocks T cell costimulatory signals before, during or after the transplant.

The present invention, as shown in independent claims 62 and 63, further provides specific dosages for the alkylating agent of the methods of the invention.

Support for pending Claim 1 is found in originally filed Claims 1, 3 and 4 and in the specification as originally filed at page 7, lines 10-18; page 16, lines 20-27; page 26, lines 1-31; page 27, lines 1-2; page 30, lines 9-16; page 31, lines 9-16; Example 1; and Example 3.

Support for pending Claim 9 is found in originally filed Claims 9, 11, 16, 17 and in the specification as originally filed at page 7, lines 10-18; page 16, lines 20-27; page 25, lines 16-20; page 26, lines 1-31; page 27, lines 1-2; page 30, lines 9-16; and page 31, lines 9-16; Example 1; and Example 3.

Support for pending Claim 34 is found in originally filed Claims 34, 35 and in the specification as originally filed at page 16, lines 20-27; page 26, lines 1-15; page 30, lines 9-16; Example 1; and Example 3.

Support for pending Claim 55 is found in originally filed Claim 42 and in the specification as originally filed at page 7, lines 10-18; page 26, lines 1-15; page 29, lines 21-22; page 31, lines 9-16; Example 1; and Example 3.

Support for pending claim 56 may be found in originally filed Claims 18, 19, 20, 27 and 29 and in the specification as originally filed at page 7, lines 10-18; page 11, lines 20-21; page 19, lines 6-19; page 26, lines 1-15; page 29, lines 21-22; page 31, lines 9-16; SEQ ID NO: 4; Figure 15; Example 1; and Example 3.

Support for pending claim 62 may be found in originally filed Claim 34 and in the specification as originally filed at page 7, lines 10-18; page 16, lines 20-27; Example 1 (page 47, lines 10-11); and Example 3.

Support for pending claim 63 may be found in originally filed Claims 1 and 3 and in the specification as originally filed at page 7, lines 10-18; page 16, lines 20-27; page 26, lines 1-31; page 27, lines 1-2; Example 1 (page 47, lines 10-11); and Example 3.

G. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

There are six (6) issues under appeal regarding (a) priority (item 4 of the March 29, 2007, Office Action), (b) rejections under 35 U.S.C. §112, first paragraph (items 5 and 6 of the March 29, 2007, Office Action) and (c) rejections under 35 U.S.C. §103(a) (items 7, 8 and 9 of the March 29, 2007, Office Action) for which applicants and the Office do not agree and respectfully request reconsideration and withdrawal.

Priority

At item 4 (pages 3-4 of the March 29, 2007, Office Action) the Office maintained the position that the filing date of the instant claims is deemed to be the filing date of priority application U.S. Serial No. 60/303,142, filed July 5, 2001, rather than priority application U.S. Serial No. 60/264,528, filed January 26, 2001.

35 U.S.C. §112, first paragraph

A. At item 5 (pages 4-7 of the March 29, 2007, Office Action), the Office maintained the rejection of claims 47-48 alleging that while the specification is enabling for the specific mutant CTLA4 molecules, such as the L104EA29YIg molecule disclosed in the specification as filed, it does not reasonably provide enablement for any "CTLA4 mutant molecule," to be employed as an immunosuppressive agent in the instant claimed methods.

B. At item 6 (page 8 of the March 29, 2007, Office Action), the Office maintained the rejection of claims 67-70 under 35 U.S.C. §112, first paragraph, as containing subject matter not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. The Office is requiring Applicants to provide assurance that the ATCC deposits of the biological materials be made readily obtainable to the public. Additionally, the Office is requiring the Applicants to amend the specification to recite the date of deposit and the complete name and address of the depository.

35 U.S.C. §103(a)

A. At item 7 (pages 9-16 of the March 29, 2007, Office Action), the Office maintained the rejection of claims 1-6, 9-13, 17, 30, 33-37, 44-52, 54-63 and rejected claim 64 as allegedly unpatentable over:

(1) Sykes et al. (U.S. Patent No. 6,514,513) in view of

(2) art known practice and modes of administration of alkylating agents such as busulfan/cyclophosphamide at various times to meet the needs of the patients, as acknowledged on pages 26-27 of the originally-filed specification and *as evidenced by*:

(i) Andersson et al. (U.S. Patent Nos. 5,430,057 and 5,559,148);

(ii) Hassan et al. (Blood, 1994, 84:2144-2150);

(iii) The Merck Manual of Diagnosis and Therapy, 17th Ed. (pages 1067-1074);

(iv) Shichi et al. (U.S. Patent No. 4,843,092);

(v) Strom et al. (Therapeutic Immunology, 1996, pages 451-456);

(vi) Sykes et al. (Nature Medicine, 1997, 3:783-787);

(vii) Wekerle et al. (J Exp Med, 1998, 187:2037-2044); and

(viii) Slattery et al. (Therapeutic Drug Monitoring, 1998, 20:543-549).

B. At item 8 (pages 17-18 of the March 29, 2007, Office Action), the Office maintained the rejection of claims 1, 9 and 33 as allegedly unpatentable over:

(1) Sykes et al. (U.S. Patent No. 6,514,513) in view of

(2) A(2)(i)-(viii) above and in view of

(3) Larsen et al. (U.S Patent No. 5,916,560).

C. At item 9 (pages 18-19 of the March 29, 2007, Office Action), the Office maintained the rejection of claims 1, 5, 6, 9-10, 12-23, 30, 34, 36-37, 44-52, 54-60, 62-63 and rejected claims 64-74 as allegedly unpatentable over:

(1) Sykes et al. (U.S. Patent No. 6,514,513) in view of

(2) A(2)(i)-(viii) above and in view of

(3) Peach et al. (US 2002/0182211).

H. ARGUMENTS

ITEM 4: PRIORITY

At item 4 (page 3 of the March 29, 2007, Office Action), the Office takes the position that the filing date of the instant claims is deemed to be the filing date of priority application U.S. Serial No. 60/303,142, filed July 5, 2001, rather than the filing date of priority application U.S. Serial No. 60/264,528 filed January 26, 2001.

The Office reiterates its allegation that the priority application, U.S. Serial No. 60/264,528, filed January 26, 2001, does not provide sufficient written description for

(a) "administering TDBM before, during and/or after a solid organ or tissue/cellular transplant";

(b) "subsequently administering an alkylating agent (including busulfan)"; or

(c) "administering an immunosuppressive composition before, during and/or after a solid organ or tissue/cellular transplant", as currently claimed.

Applicants' Argument

Applicants respectfully disagree that the priority application U.S. Serial No. 60/264,528, filed January 26, 2001, does not sufficiently describe the specific specification passages of (a)-(c) *supra* for reasons of record.

Contrary to the Office's position that parent provisional application U.S. Serial No. 60/264,528 filed January 26, 2001, only discloses a "single dose of busulfan prior to the transplantation (i.e. intravenous infusion) of T cell-depleted bone marrow cells", data supporting passages (a)-(c) *supra* can be found in said parent provisional application as follows.

Applicants' support for passage (a) ("administering TDBM before, during and/or after a solid organ or tissue/cellular transplant") may be found in the skin graft experiments, at pages 4 and 5 of U.S. Serial No. 60/264,528. These experiments clearly describe administering TDBM on day 0 and on day 6 (page 4, line 12). The skin graft was done on day 0 (page 4, line 13). The data is shown in Figure 2A. Figure 2A clearly supports a claim of administering TDBM during and after a transplant. The claim of administering TDBM before the transplant is shown in the experiments described on pages 4-5 of U.S. Serial No. 60/264,528, in which the mice which were administered TDBM on days 0 and 6 (page 4, line 12) and were re-challenged with skin transplants 100 days after the original transplant/protocol (page 5, lines 5 and 6).

Applicants' support for passage (b) ("subsequently administering an alkylating agent") (e.g., busulfan) is in the skin graft experiments (page 4, lines 8-13 and page 2, lines 18-23) and data is shown in Figure 2 of U.S. Serial No. 60/264,528. Herein, Applicants describe using busulfan on day 5. Thus, in the skin graft experiments, TDBM cells are administered on day 0, along with the skin graft, busulfan is administered on day 5, and TDBM cells are administered on day 6.

Regarding Applicants' support for passage (c) ("administering an immunosuppressive composition before, during and/or after a solid organ or tissue/cellular transplant"), the immunosuppressive composition (e.g., costimulatory blockade (CB) comprising a combination of a first ligand that interferes with binding of CD28 to either CD80 or CD86, and a second ligand that interferes with binding of CD154 to CD40), is used in the skin graft experiments described (page 4, lines 8-12 and page 2, lines 21-22) of U.S. Serial No. 60/264,528. The data is shown in Figure 2. The co-stimulatory blockade is described at page 2, line 22, and page 4, lines 11-13 of U.S. Serial No. 60/264,528, as being administered on days 0, 2, 4, 6, 14, and 28. As transplantation was performed on day 0 (page 4, line 13) and day 100 (page 5, lines 5-6), the use of the terms "before, during and/or after" is appropriate.

Accordingly, U.S. Serial No. 60/264,528 supports the instant claims and Applicants are entitled to the January 26, 2001, filing date. Applicants request that the Office withdraw the rejection.

ITEM 5: REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH

At item 5 (pages 4-7 of the March 29, 2007, Office Action) the Office maintained the rejection of claims 47-48 alleging that while the specification is enabling for the specific mutant CTLA4 molecules, such as the L104EA29YIg molecule disclosed in the specification as filed, it does not reasonably provide enablement for any "CTLA4 mutant molecule," to be employed as an immunosuppressive agent in the instant claimed methods.

Applicants' Argument

Applicants respectfully disagree for reasons of record.

Applicants traverse the rejection because Applicants provide methods for screening CTLA4 mutants for their binding capacity (Example 9, pages 71-83, of the instant application). Further, Applicants provide examples of over thirty mutant molecules (Tables I and II at pages 82 and 83 and on page 70, lines 22-25), including their entire nucleotide sequences, and described the required functions for other members of the class of proteins (page 75, line 28-page 78, line 24, page 79, line 20-page 81).

35 U.S.C. § 112, first paragraph, requires Applicants to teach how to make and use the invention, without undue experimentation. The law is clear. Applicants are not required to disclose every species encompassed by the claims (In re Angstadt and Griffin, 537 F.2d 498, 190 USPQ 214, 218 (CCPA 1976)). Moreover, despite the fact that Applicants do not disclose every known CTLA4 mutant molecule, the identification of other species in the class would not entail undue experimentation, because Applicants' disclosure

outlines a number of different assays for the identification of CTLA4 mutant molecule as claimed. Practice of the claimed invention does not require undue experimentation.

In view of the preceding remarks, Applicants respectfully request that the Office reconsider and withdraw the rejection set forth in the Office Action.

ITEM 6: REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH

At item 6 (page 8 of the March 29, 2007 Office Action), the Office maintained the rejection of claims 67-70 under 35 U.S.C. §112, first paragraph, as containing subject matter not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. The Office is requiring Applicants to provide assurance that the ATCC deposits of the biological materials be made readily obtainable to the public. Additionally, the Office is requiring the Applicants to amend the specification to recite the date of deposit and the complete name and address of the depository.

Applicants' Argument

Claims 69-70 are directed to a cell line deposited as ATCC 10762.

The specification as originally filed states at page 19, lines 27 to page 20, line 2, that "(DNA encoding CTLA4Ig was deposited on May 31, 1991 with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209 under the provisions of the Budapest Treaty, and has been accorded ATCC accession number ATCC 68629; Linsley, P., et al., 1994 *Immunity* 1:793-80). CTLA4Ig-24, a Chinese Hamster Ovary (CHO) cell line expressing CTLA4Ig was deposited on May 31, 1991 with ATCC identification number CRL-10762)."

The originally filed specification already recites the date of deposit and the complete name and address of the depository as required by the Office. However, on December 13, 2006, to further prosecution, Applicants amended the specification to clarify that ATCC CRL-10762 was deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209 under the provisions of the Budapest Treaty (page 2 of Applicants' amendment submitted to the Office on December 13, 2006).

Also, on December 13, 2006, Applicants amended Claims 69 and 70 to clarify the ATCC identification number from "ATCC 10762" to "ATCC CRL-10762".

Statement of ATCC Deposit:

As previously stated on December 13, 2006 (at page 21 of Applicants' amendment), Applicants' patent representative maintains that the cell line with ATCC Accession No. CRL-10762 was deposited on May 31, 1991, pursuant to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure with the Patent Culture Depository of the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209 U.S.A. Applicants provide a copy of the Receipt Forms issued by the American Type Culture Collection, confirming deposit of CRL-10762 and ATCC 68629 (Evidence Appendices 1 and 2).

Also, Applicants stated in the December 13, 2006, amendment (at page 21) that during the pendency of the present application, access to the ATCC deposits will be afforded to one determined by the Commissioner to be entitled thereto under 35 U.S.C. §§1.14 and 122, and all restrictions on the availability to the public of the materials deposited under ATCC Accession Nos. 68629 and CRL-10762 will be irrevocably removed upon the issuance of a patent from the present application. Furthermore, the above deposits will be maintained by the ATCC for a period of 30 years from the date of deposit or at least 5 years after the last request for a sample of the deposited material, whichever is longer.

Where the ATCC cannot furnish samples of the above deposits for any reason, Applicants shall make a replacement deposit, of the material which was originally deposited, within three months of receiving notification that the ATCC cannot furnish samples.

Additionally, pursuant to the Office's requirement on page 8 of the March 29, 2007 Office Action, Applicants state that "all restrictions imposed by the depositor on the availability to the public of the deposited material will be irrevocably removed upon the granting of a patent in U.S. patent applications."

Applicants also note that material deposited under ATCC Accession No. 68629 is already available to the public due to the grant of U.S. Patent 5,885,796 which contains claims directed to the material.

In view of the passage in the originally filed specification cited *supra*, Applicants statements *supra* and the December 13, 2006 amendments to the specification and Claims 69-70, Applicants respectfully request that the Patent Office reconsider and withdraw the rejection of Claims 67-70, under 35 U.S.C. §112, first paragraph.

REJECTION UNDER 35 U.S.C. §103(a)

(A) At item 7 (pages 9-16 of the March 29, 2007, Office Action), the Office maintained the rejection of claims 1-6, 9-13, 17, 30, 33-37, 44-52, 54-63 and rejected claim 64 as allegedly unpatentable over:

(1) Sykes et al. (U.S. Patent No. 6,514,513) in view of

(2) art known practice and modes of administration of alkylating agents such as busulfan/cyclophosphamide at various times to meet the needs of the patients, as acknowledged on pages 26-27 of the originally-filed specification *as evidenced by*:

(i) Andersson et al. (U.S Patent Nos. 5,430,057 and 5,559,148);

(ii) Hassan et al. (Blood, 1994, 84:2144-2150);

(iii) The Merck Manual of Diagnosis and Therapy, 17th Ed. (pages 1067-1074);

(iv) Shichi et al. (U.S. Patent No. 4,843,092);

(v) Strom et al. (Therapeutic Immunology, 1996, pages 451-456);

(vi) Sykes et al. (Nature Medicine, 1997, 3:783-787);

(vii) Wekerle et al. (J Exp Med, 1998, 187:2037-2044); and

(viii) Slattery et al. (Therapeutic Drug Monitoring, 1998, 20:543-549).

(B) At item 8 (pages 17-18 of the March 29, 2007, Office Action), the Office maintained the rejection of claims 1, 9 and 33 as allegedly unpatentable over Sykes et al. (U.S. Patent No. 6,514,513) in view of A(2)(i)-(viii) above and in view of Larsen et al. (U.S Patent No. 5,916,560).

(C) At item 9 (pages 18-19 of the March 29, 2007, Office Action), the Office maintained the rejection of claims 1, 5, 6, 9-10, 12-23, 30, 34, 36-37, 44-52, 54-60, 62-63 and rejected claims 64-74 as allegedly unpatentable over Sykes et al. (U.S. Patent No. 6,514,513) in view of A(2)(i)-(viii) above and in view of Peach et al. (US 2002/0182211)

Applicants' Argument

Applicants respectfully disagree.

The Legal Standard for 35 U.S.C. §103

As stated in MPEP §2142, three (3) criteria must be met to establish a *prima facie* case of obviousness:

First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the

> art, to modify the references or to combine reference teachings. *Second*, there must be a reasonable expectation of success. *Finally*, the prior art reference must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based upon applicants' disclosure.[1]

The teaching or suggestion to make the claimed combination, and the reasonable expectation of success, must both be found in the prior art, not in the Applicants' disclosure (In re Vaeck, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991)).

Obviousness is a question of law based on findings of underlying facts relating to the prior art, the skill of the artisan, and objective considerations. See Graham v. John Deere Co., 383 U.S. 1, 17, 86 S.Ct. 684, 694, 148 USPQ 459, 467 (1966). To establish a prima facie case of obviousness based on a combination of the content of various references, there must be some teaching, suggestion or motivation in the prior art to make the specific combination that was made by the applicant. In re Raynes, 7 F.3d 1037, 1039, 28 USPQ2d 1630, 1631 (Fed. Cir. 1993); In re Oetiker, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1445 (Fed. Cir. 1992). Obviousness can not be established by hindsight combination to produce the claimed invention. In re Gorman, 933 F.2d 982, 986, 18 USPQ2d 1885, 1888 (Fed. Cir. 1991). As discussed in Interconnect Planning Corp. v. Feil, 774 F.2d 1132, 1143, 227 USPQ 543, 551 (Fed. Cir. 1985), it is the prior art itself, and not the applicant's achievement, that must establish the obviousness of the combination.

The teachings of the references, their relatedness to the field of the applicant's endeavor, and the knowledge of persons of ordinary skill in the field of the invention, are all relevant considerations. See In re Oetiker, 977 F.2d at 1447, 24 USPQ2d at 1445-46; In re Gorman, 933 F.2d at 986-87, 18 USPQ2d at 1888; In re Young, 927 F.2d 588, 591, 18 USPQ2d 1089, 1091 (Fed. Cir. 1991). When the references are in the same field as that of

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[1] MPEP §2142, *citing* In re Vaeck, 947 F.2d 488, 20 U.S.P.Q.2d 1438 (Fed. Cir. 1991).

the applicant's invention, knowledge thereof is presumed. However, the test of whether it would have been obvious to select specific teachings and combine them, as did the applicant, must still be met by identification of some suggestion, teaching, or motivation in the prior art, arising from what the prior art would have taught a person of ordinary skill in the field of the invention. In re Fine, 837 F.2d 1071, 1075, 5 USPQ2d 1596, 1600 (Fed. Cir. 1988).

The Office Has Not Established A *Prima Facie* Case Of Obviousness

The Office has not established a *prima facie* case of obviousness because not all of the three necessary criteria have been met. Therefore, as discussed *infra*, the pending claims are patentable over the cited references.

Applicants' Invention

The present invention as shown in independent claims 1, 9, 34, 55, 56, 62 and 63 provides methods for inhibiting or reducing rejection of a solid organ or tissue/cellular transplant in a subject comprising the following sequence of steps: administering T cell depleted bone marrow cells to the subject before, during or after the solid organ or tissue/cellular transplant; administering an alkylating agent (e.g., busulfan) to the subject in an amount that facilitates mixed chimerism; administering a subsequent dose of T cell depleted bone marrow; and administering an immunosuppressive composition that blocks T cell costimulatory signals in the subject before, during or after the transplant.

Also, the present invention as shown in independent claims 62 and 63 further provides specific dosages for the alkylating agent of the methods.

A Discussion of the References Cited by the Office

Sykes, U.S. Patent No. 6,514,513 ('513)

Sykes discloses a method of promoting graft acceptance (e.g., skin graft), by a recipient mammal, wherein the graft is from a donor mammal of a second species. The method includes: administering to the recipient, an inhibitor, (e.g., either CTLA4Ig or anti-CD40 ligand mAB); administering low dose whole body irradiation; introducing hematopoietic stem cells (e.g., a bone marrow preparation) into the recipient mammal; and preferably, implanting the graft in the recipient. The hematopoietic cells are believed to prepare the recipient for the graft that follows, by inducing tolerance at both the B-cell and T-cell levels (Sykes '513 at column 1, lines 49-52).

Sykes uses irradiation in the methods described (Sykes '513 at column 22, lines 36-40; column 23, lines 15-19; column 24, lines 34-36; column 25, lines 9-12; and column 27, lines 52-55) but also suggests that busulfan may be used in lieu of irradiation, to create hematopoietic space. However, this suggestion is merely a wish. There is no reasonable expectation of success if busulfan is substituted for whole body irradiation. This is because there is no direct correlation between the irradiation dosage and the busulfan dosage administered, to facilitate mixed hematopoietic chimerism. In fact, Hassan et al., discussed *infra*, teach that total body irradiation is superior to busulfan in terms of patient survival.

Sykes fails to teach the use of busulfan together with other agents of the claimed methods for facilitating mixed hematopoietic chimerism, and the effective dosage of busulfan for such use. Additionally, Sykes fails to teach the therapeutic sequence of the claimed invention as disclosed in independent claims 1, 9, 34, 55, 56, 62 and 63. The claimed methods requires: T cell depleted bone marrow administered to a subject; an alkylating agent administered to a subject after the bone marrow; additional T cell depleted bone

marrow administered to a subject after the alkylating agent; and administration of a costimulatory blockade to a subject.

The combination of Sykes '513 and the additional references (discussed *infra*) cited by the Office does not rectify the deficiencies in Sykes and does not render the claimed invention obvious.

Andersson et al., U.S Patent Nos. 5,430,057 ('057) and. 5,559,148 ('148)

Patents '057 and '148 have identical disclosures, since the '148 patent is a continuation of the '057 patent. Accordingly, the '057 and the '148 patents are discussed together herein. The '057 and the '148 patents provide methods for use of parenteral formulation of busulfan, in the clinical treatment of human neoplasms, with therapy based on parenteral preparation alone, or in combination with other cytotoxic agent(s). Additionally, these patents provide formulations to increase solubility of busulfan, design of a chemically stable formulation of busulfan that is suitable for parenteral administration, and techniques to extract busulfan from blood, as well as pharmacokinetics of commercially available busulfan, and busulfan when solubilized in polyethylene glycol.

The '057 and the '148 patents fail to teach what the primary reference fails to teach, namely, the use of alkylating agents, e.g., busulfan, together with other agents of the claimed methods for facilitating mixed hematopoietic chimerism, and the effective dosage of busulfan for such use. Moreover, these patents fail to teach or suggest the use of busulfan for inhibition or reduction of rejection of solid organ transplants. Accordingly, the combination of the Sykes ('513) and the '057 and '148 patents does not render obvious the claimed methods.

Slattery et al., Therapeutic Drug Monitoring, 1998, 20:543-549

Slattery et al. provide methods for use of busulfan, to ablate marrow before hematopoietic stem cell transplantation, and the use of high levels of busulfan, in combination with cyclophosphamide, to treat patients with chronic myeloid leukemia. Further, Slattery et al. state that the therapeutic window for busulfan is narrow, and disease and graft-source dependent (abstract).

Slattery et al. fail to teach what the primary reference fails to teach, namely, the use of busulfan together with other agents of the claimed methods, for facilitating mixed hematopoietic chimerism, and the effective dosage of busulfan for such use. Further, Slattery et al. do not teach or suggest the use of busulfan for inhibition or reduction of rejection of solid organ transplants. Accordingly, the combination of the Sykes ('513) and Slattery et al. does not render obvious the claimed methods.

Hassan et al., Blood, 1994, 84:2144-2150

Hassan et al. provides methods for use of busulfan in patients only undergoing bone marrow transplantation and evaluates the bioavailability of busulfan. Additionally, Hassan et al. note that although busulfan has been introduced as an alternative to total body irradiation (TBI), TBI treatment of patients conferred a survival advantage over busulfan treated patients (page 2144, column 1, paragraph 2).

Hassan et al. teach that busulfan has a higher mortality rate compared to TBI. Accordingly, Hassan et al. teach away from the claimed methods. In view of teaching of Hassan et al., the combination of references cited by the Office in its rejection of the claims fails to render the claimed methods obvious.

The Merck Manual of Diagnosis and Therapy, 17th Ed., edited by Beers et al., 1999

The Merck Manual (pages 1067-1074) teaches an overview of biology related to transplantation including the immunobiology of rejection, components of tissue compatibility and immunosuppression. Immunosuppressive drugs mentioned in the Merck Manual include corticosteroids, azathioprine, cyclophosphamide, cyclosporine and tacrolimus. Other immunosuppressive factors mentioned in the Merck Manual include monoclonal antibodies and irradiation.

The Merck Manual fails to teach what the primary reference ('513) fails to teach, namely, the use of alkylating agents, e.g., busulfan, together with other agents of the claimed methods, for facilitating mixed hematopoietic chimerism, and the effective dosage of busulfan for such use. Further, the Merck Manual does not teach or suggest the use of busulfan for inhibition or reduction of rejection of solid organ transplants. Accordingly, the combination of the '513 and the Merck Manual references does not render obvious the claimed methods.

Shichi et al., U.S. Patent No. 4,843,092

Shichi et al. teach an immunosuppressive agent comprising macrolide antibiotic(s) for suppressing rejection after organ transplantation and as an agent for treating immune diseases. The background section of Shichi et al. mentions that "[a]s immunosuppressive agents, there are known alkylating agents such cyclophosphamide" which can be used as agents for suppressing rejection which may occur after transplantation. Shichi et al. does not teach how to use cyclophosphamide (i.e., does not teach any methods of administration nor any dosages by itself or in combination with other agents) much less teach the use of busulfan.

Shichi et al. fail to teach what the primary reference ('513) fails to teach, namely, the use of alkylating agent, e.g., busulfan, together with other agents of the claimed methods, for

facilitating mixed hematopoietic chimerism, and the effective dosage of an alkylating agent, e.g., busulfan, for such use. Further, Shichi et al. do not teach or suggest the use of an alkylating agent, e.g., busulfan, for inhibition or reduction of rejection of solid organ transplants. Accordingly, the combination of the '513 and Shichi et al. references does not render obvious the claimed methods.

Strom et al., Therapeutic Immunology edited by Austen et al., 1996

Strom et al. teach the use of multiple agents simultaneously, with each agent directed at a different molecular target, for immunosuppressive therapy. The agents cited by Strom et al. to be used in combination are: cyclosporine, tacrolimus (FK506), corticosteroids, azathioprine, mycophenolate mofetil, OKT3 monoclonal antibody, anti-IL-2 antibody, anti-IL-2 receptor antibody, anti-adhesion molecule antibody and rapamycin.

Strom et al. fail to teach what the primary reference fails to teach, namely, the use of an alkylating agent, e.g., busulfan, together with other agents of the claimed methods for facilitating mixed hematopoietic chimerism and tolerance induction, and the effective dosage of an alkylating agent, e.g., busulfan, for such use. Moreover, Strom et al. fail to teach or suggest the use of an alkylating agent, e.g., busulfan, for inhibition or reduction of rejection of solid organ transplants. Accordingly, the combination of the Sykes ('513) and the Strom et al. references does not render obvious the claimed methods.

Sykes et al., Nature Medicine 3:783-787, 1997

Sykes et al. teach donor specific T-cell tolerance induced by administering to a murine subject: 1) depleting anti-CD4 and anti-CD8 monoclonal antibodies to remove the host immune barriers to T cell allo-engraftment, 2) local thymic irradiation to produce space in the thymic compartment, and 3) a high dose of MHC-mismatched bone marrow cells.

Sykes et al. fail to teach what the primary reference ('513) fails to teach, namely, the use of an alkylating agent, e.g., busulfan, together with other agents of the claimed methods, for facilitating mixed hematopoietic chimerism, and the effective dosage of an alkylating agent, e.g., busulfan, for such use. Sykes et al. do not teach or suggest the use of an alkylating agent, e.g., busulfan, for inhibition or reduction of rejection of solid organ transplants. Accordingly, the combination of the '513 and Sykes et al., references does not render obvious the claimed methods.

Wekerle et al., J Exp Med, 187:2037-2044, 1998

Wekerle et al. teach induction of transplantation tolerance by administering to a murine subject: 1) single injections of anti-CD40 ligand antibody and CTLA4Ig, 2) whole body irradiation, and 3) MHC-mismatched allogeneic bone marrow transplantation.

However, Wekerle et al. fail to teach what the primary reference ('513) fails to teach, namely, the use of an alkylating agent, e.g., busulfan, together with other agents of the claimed methods, for facilitating mixed hematopoietic chimerism, and the effective dosage of an alkylating agent, e.g., busulfan, for such use. Moreover, Wekerle et al. fail to teach or suggest the use of an alkylating agent, e.g., busulfan, for inhibition or reduction of rejection of solid organ transplants. Accordingly, the combination of the '513 and Wekerle et al. references does not render obvious the claimed methods.

THE LEGAL STANDARD HAS NOT BEEN MET BY THE OFFICE

The references in combination do not teach all of the claimed steps

The Office asserts that the claimed method is an obvious modification of the Sykes reference. However, as discussed *supra*, the prior art references in combination does not teach or suggest all of the claim limitations, in the order claimed, namely, steps a-d of claims 1, 9, 34, 55, 56, 62 or 63.

Moreover, Hassan et al. teach away from the claimed method. Thus the combination of the prior art references does not render obvious the claimed method.

There was no suggestion to modify the prior art in order to obtain the claimed invention.

The Office's statement that it was within the skill in the art to make the modifications necessary to advance from the prior art to the claimed method is similar to an erroneous statement made in Ex parte Levengood.[2] In Levengood, the examiner stated that because the various aspects of the claimed process were individually known in the art (in the instant case, this is not true), the modifications of a prior art process necessary to arrive at the claimed invention were "well within the ordinary skill of the art at the time the claimed invention was made."[3]

The Board of Patent Appeals and Interferences reversed the examiner's rejection because it was based on the wrong standard of obviousness: "At best, the examiner's comments regarding obviousness amount to an assertion that one of ordinary skill in the relevant art would have been able to arrive at appellant's invention because he had the necessary skills to carry out the requisite process steps. This is an inappropriate standard for obviousness. . . . That which is within the capabilities of one skilled in the art is not synonymous with obviousness."[4]

The Office's current reliance on what was within the skill in the art to support the obviousness of the modifications separating the prior art from the claimed invention is likewise an erroneous basis for finding the invention *prima facie* obvious over the cited art.

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[2] 28 USPQ2d 1300 (BPAI 1993).
[3] *Id.* at 1301.
[4] *Id.* (citations omitted).

To establish a *prima facie* case of obviousness, the Office must present evidence that one skilled in the art would have been led to arrive at the claimed invention.[5] Mere unsupported arguments cannot take the place of evidence.[6]

In this regard, Sykes ('513) merely suggests that other methods of creating hematopoietic space, e.g., administering hematopoietic space creating antibodies or drugs, e.g., cyclophasmide or busulfan, to the recipient, can be used (Sykes '513 at column 5, lines 3-5). Without more, this statement cannot suggest the claimed invention. Merely desiring an end result does not constitute a specific modification of the prior art.

Even if the prior art were combined, there would have been no predictability or reasonable expectation of success in achieving the claimed invention.

It would not be enough to imply that, given the capabilities of those skilled in the art, it would have been obvious to try the claimed invention. In In re O'Farrell, the Federal Circuit gave examples of what would be obvious to try, but not obvious under 35 U.S.C. §103: "to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it."[7] O'Farrell clarified the additional requirement for a reasonable expectation of success.

Sykes ('513) provides only a cursory statement of replacing irradiation with busulfan as a preparative regimen for bone marrow transplants (BMT). This is very different from the claimed methods of inhibiting solid organ transplants. None of the cited references, alone, or in combination, provides guidance for modifying the methods to achieve therapeutically effective methods as claimed. In fact, Hassan et al. teach that total body irradiation is superior to busulfan in terms of patient survival. Moreover, there was no reason to believe that busulfan dosages of the art as a preparative regimen for BMT

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[5] *Id.*
[6] In re Wiseman, 596 F.2d 1019, 201 USPQ 658, 661 (CCPA 1979).
[7] 853 F.2d 894, 7 USPQ2d 1673, 1681 (Fed. Cir. 1988).

would be extrapolatable for busulfan dosages for facilitating MHC in connection with solid organ transplants. Such cursory statements are not equivalent to a reasonable expectation of success because there was no direction or guidance on how to proceed to achieve the prophetic goal based on the references.

Additionally, Sykes does not teach any therapeutic sequence. The claimed method requires: administration of T cell depleted bone marrow to a subject; administration of an alkylating agent after the T cell depleted bone marrow to a subject; administration of additional T cell depleted bone marrow after the alkylating agent to a subject; and administering an immunosuppressive composition that blocks T cell costimulatory signals in the subject.

Sykes ('513) fails to teach the use of alkylating agents, e.g., busulfan, together with other agents of the claimed methods for facilitating mixed hematopoietic chimerism, and the effective dosage of alkylating agents, e.g., busulfan, for such use. Additionally, Sykes ('513) fails to teach the therapeutic sequence of the claimed method.

In In re Gangadharam, the Federal Circuit reversed an obviousness rejection maintained by the Board of Patent Appeals and Interferences on the basis of a single prior art reference because the Patent and Trademark Office (PTO) had failed to meet its burden of proving a *prima facie* case of obviousness.[8] The single cited reference stated that the result reported therein "offers a hopeful lead" for the therapeutic use claimed in the later application. The Federal Circuit stated that the Board's attempt to base their finding of a reasonable expectation of success for the claimed use on the one prior art reference "fell woefully short of its burden."[9]

As in Gangadharam, the Office in the present case is basing the rejection under Section 103, of the claimed methods on the hopeful lead provided by the prior art. Like

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[8] 889 F.2d 1101, 13 USPQ2d 1568 (Fed. Cir. 1989).
[9] *Id.* at 1569.

Gangadharam, the Office's attempt to base an obviousness rejection on the cited reference, i.e., Sykes ('513), falls woefully short of its burden because data using irradiation as a preparative regimen cannot teach the claimed method, for inhibiting rejection of a solid organ transplant in a subject. In fact, art cited by the Office teaches that radiation treatment is not equivalent to busulfan treatment (Hassan et al.).

The Office has not provided evidence that the prior art teaches or suggests *as a whole* the claimed methods. The claimed methods cannot be obvious over the cited references, because there was no suggestion regarding how to modify the prior art, in order to achieve the claimed methods. Moreover, even if it were obvious to try the combination of elements claimed, much less the specified sequence of administering the elements, without a reasonable expectation of success, a *prima facie* case of obviousness cannot be made. It is therefore, respectfully requested that the rejection under 35 U.S.C. §103, be withdrawn, and that the claims be allowed.

THE CLAIMED INVENTION POSSESSES UNEXPECTED ADVANTAGES THAT THE CITED REFERENCES DOES NOT TEACH

Applicants respectfully contend that the cited references do not render the claimed invention *prima facie* obvious. Furthermore, the alleged obviousness is rebutted by evidence of unexpected properties of the claimed invention (In re Davies, 475 F.2d 667, 177 U.S.P.Q. 381 (CCPA 1973)).

In addition to Applicants' previous showing, Applicants also previously provided post filing confirmatory data (in an Amendment dated March 24, 2006) showing that the methods of the invention possess superior properties. Specifically, Applicants provided the following:

1. L. Kean et al. (Evidence Appendix 3)

   Here, the authors show that nonmyeloablative preconditioning with busulfan (20mg/kg) coupled with costimulation blockade (CTLA4-Ig and anti-CD40L) can safely produce stable white blood cell (WBC) mixed chimerism and total replacement of the peripheral red cell compartment, resulting in a phenotypic cure of murine SCD. Furthermore, this cure is accomplished with fully major histocompatibility complex (MHC) mismatched donor marrow. Importantly, the hematologic cure that occurred with total replacement of the red cell compartment was accompanied by normalization of characteristic sickle organ pathology, indicating a total-body amelioration of disease.

2. Z. Guo et al. (Evidence Appendix 4)

   The results of these studies demonstrate that the infusion of donor bone marrow together with busulfan and costimulation blockade (anti-CD40L mAb and CTLA4-Ig) induces hematopoietic chimerism and promotes the long-term survival of intestinal allografts transplanted into mice that have completed the treatment regimen. This long-term survival is associated with donor-specific hyporesponsiveness in vitro and deletion of donor-reactive T cells in vivo.

3. N. Shirasugi et al. (Evidence Appendix 5)

   Treatment regimens consisting of costimulation bloackade CB alone (CTLA4-Ig and anti-CD40L), CB and donor bone marrow cells (BMCs), and CB and donor splenocytes (DST) promote long-term allograft survival, but do not confer robust tolerance nor prevent chronic rejection in the face of a rechallenge with a donor skin graft. In contrast, a regimen

> consisting of CTLA4-Ig, anti-CD40L, donor BMCs, and a minimally myelosuppressive dose of busulfan produced stable donor-specific tolerance, and prevented both early and late cellular infiltration and chronic allograft vasculopathy, despite the rigorous rechallenge of a donor skin graft.

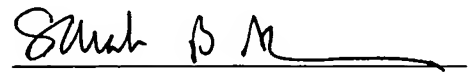
In view of the aforementioned discussion, Applicants respectfully request that the Patent Office reconsider and withdraw the rejection of the claims, under 35 U.S.C. §103.

CONCLUSION

Applicants respectfully request that the rejections be reversed and pending Claims 1, 2, 5, 6, 9, 10, 12, 13, 30, 33, 34, 36, 37, 44-52, 54-60 and 62-74 be allowed.

No fees, other than the $2590.00 fees for filing the Appeal Brief, three-month extension of time and Request for Oral Hearing, are deemed necessary in connection with the filing of this Appeal Brief. If any further fees are necessary, the Patent Office is authorized to charge any additional fee to Deposit Account No. 50-0306.

Respectfully submitted,



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I. CLAIMS APPENDIX

1. (Under Appeal) A method of inhibiting rejection of a solid organ or tissue/cellular transplant in a subject comprising:

   a) administering T cell depleted bone marrow cells to the subject before, during and/or after a solid organ or tissue/cellular-transplant;

   b) subsequently, after step (a), administering an alkylating agent to the subject in an amount that facilitates mixed hematopoietic chimerism, wherein the alkylating agent is selected from a group consisting of alkylsulfonates, nitrogen mustards, oxazaposporines, nitrosoureas, and alkylating chemotherapeutic agents;

   c) administering a second dose of T cell depleted bone marrow cells to the subject after step (b); and

   d) administering to the subject costimulatory blockade before, during and/or after the transplant, which costimulatory blockade blocks T cell costimulatory signals in the subject, and wherein the costimulatory blockade comprises a combination of a first ligand that interferes with binding of CD28 to either CD80 or CD86, and a second ligand that interferes with binding of CD154 to CD40;

   thereby inhibiting rejection of the solid organ or tissue/cellular-transplant.

2. (Under Appeal) The method of claim 1, wherein the alkylating agent is busulfan.

3-4. (Canceled)

5. (Under Appeal) The method of claim 1, wherein the first ligand is a soluble CTLA4 molecule.

6. (Under Appeal) The method of claim 1, wherein the first ligand is a CTLA4Ig.

7-8. (Canceled)

9. (Under Appeal) A method for establishing mixed hematopoietic chimerism in a subject so as to inhibit or reduce rejection of a solid organ or tissue/cellular transplant, comprising:

a) administering T cell depleted bone marrow cells to a subject having a solid organ or tissue/cellular transplant;

b) administering an alkylating agent to the subject after step (a), in an amount that facilitates mixed hematopoietic chimerism, wherein the alkylating agent is selected from a group consisting of alkylsulfonates, nitrogen mustards, oxazaposporines, nitrosoureas, and alkylating chemotherapeutic agents;

c) administering a second dose of T cell depleted bone marrow cells to the subject after step (b); and

d) administering costimulatory blockade that blocks T cell costimulatory signals in the subject before, during and/or after the transplant, wherein the costimulatory blockade comprises a combination of a first ligand that interferes with binding of CD28 to either CD80 or CD86, and a second ligand that interferes with binding of CD154 to CD40,

thereby establishing hematopoietic chimerism in the subject so as to inhibit or reduce rejection of the solid organ or tissue/cellular transplant.

10. (Under Appeal) The method of claim 9, wherein the alkylating agent is busulfan.

11. (Canceled)

12. (Under Appeal) The method of claim 9, wherein the first ligand is a soluble CTLA4 molecule.

13. (Under Appeal) The method of claim 9, wherein the first ligand is a CTLA4Ig.

14-29. (Canceled)

30. (Under Appeal) The method of claim 1 or 9, wherein one or both of the T cell depleted bone marrow so administered is administered before the transplant and wherein the busulfan is administered within any of (a) 24 hours prior to the solid organ or tissue/cellular transplant, (b) twelve hours prior to the solid organ or tissue/cellular transplant, or (c) six hours prior to the solid organ or tissue/cellular transplant.

31-32. (Canceled)

33. (Under Appeal) The method of claim 1 or 9, wherein the transplant is a skin graft.

34. (Under Appeal) A method of reducing rejection of a solid organ or tissue/cellular transplant in a subject in need thereof comprising:

a) administering a first dose of T cell depleted bone marrow cells and costimulatory blockade to a subject;

b) placement of an organ or tissue/cellular transplant to the subject before, during and/or after the administration of the costimulatory blockade;

c) administering busulfan to the subject in an amount that facilitates mixed chimerism after step (a); and

d) administering a second dose of T cell depleted bone marrow cells and costimulatory blockade after step (c), wherein the costimulatory blockade is a combination of a first ligand that interferes with binding of CD28 to either CD80 or CD86, and a second ligand that interferes with binding of CD154 to CD40,

thereby reducing rejection of the solid organ or tissue/cellular transplant.

35. (Canceled)

36. (Under Appeal) The method of claim 34, wherein the first ligand is a soluble CTLA4 molecule.

37. (Under Appeal) The method of claim 34, wherein the first ligand is a CTLA4Ig.

38-43. (Canceled)

44. (Under Appeal) The method of claim 5, 12, or 36, wherein the soluble CTLA4 molecule comprises an extracellular domain of CTLA4 which binds CD80 and/or CD86.

45. (Under Appeal) The method of claim 44, wherein the extracellular domain of CTLA4 has an amino acid sequence which begins with methionine at position 27 and ends with aspartic acid at position 150 as shown in SEQ ID NO:14, or which begins with alanine at position 26 and ends with aspartic acid at position 150 as shown in SEQ ID NO:14.

46. (Under Appeal) The method of claim 6, 13 or 37, wherein the CTLA4Ig comprises an amino acid sequence which begins with methionine at position 27 and ends with lysine at position 383 as shown in SEQ ID NO:14, or which begins with alanine at position 26 and ends with lysine at position 383 as shown in SEQ ID NO:14.

47. (Under Appeal) The method of claim 5, 12, or 36, wherein the soluble CTLA4 molecule is a soluble CTLA4 mutant molecule that interferes with the binding of CD28 to CD80 and/or CD86.

48. (Under Appeal) The method of claim 47, wherein the soluble CTLA4 mutant molecule comprises a mutated extracellular domain of CTLA4 which binds CD80 and/or CD86.

49. (Under Appeal) The method of claim 48, wherein the mutated extracellular domain of CTLA4 has an amino acid sequence which begins with methionine at position 27 and ends with aspartic acid at position 150 as shown in SEQ ID NO:4, or which begins with alanine at position 26 and ends with aspartic acid at position 150 as shown in SEQ ID NO:4.

50. (Under Appeal) The method of claim 47, wherein the soluble CTLA4 mutant molecule comprises an amino acid sequence which begins with methionine at position 27 and ends with lysine at position 383 as shown in SEQ ID NO:4, or which begins with alanine at position 26 and ends with lysine at position 383 as shown in SEQ ID NO:4.

51. (Under Appeal) The method of claim 1, 9 or 34, wherein the second ligand is a ligand for CD40.

52. (Under Appeal) The method of claim 51, wherein the ligand for CD40 is an anti-CD40 antibody or fragment thereof.

53. (Canceled)

54. (Under Appeal) The method of claim 1, 9 or 34, wherein the first ligand is a soluble CTLA4 molecule and the second ligand is an anti-CD40 Ab or a fragment thereof.

55. (Under Appeal) A method of inhibiting rejection of a solid organ or tissue/cellular transplant in a subject comprising

a) administering T cell depleted bone marrow cells to the subject;

b) subsequently, after step (a), administering busulfan to the subject in an amount that facilitates mixed hematopoietic chimerism;

c) administering a second dose of T cell depleted bone marrow cells to the subject after step (b); and

d) administering CTLA4Ig and an anti-CD40 antibody or fragment thereof to the subject before, during and/or after the solid organ or tissue/cellular transplant,

thereby inhibiting rejection of the solid organ or tissue/cellular transplant.

56. (Under Appeal) A method of inhibiting rejection of a solid organ or tissue/cellular transplant in a subject having a transplanted tissue comprising

a) administering T cell depleted bone marrow cells to a subject;

b) subsequently, after step (a), administering busulfan to the subject in an amount that facilitates mixed hematopoietic chimerism;

c) administering a second dose of T cell depleted bone marrow cells to the subject after step (b); and

d) administering a soluble CTLA4 mutant molecule comprising an amino acid which begins with methionine at position 27 and ends with lysine at position 383 as shown in SEQ ID NO:4, or which begins with alanine at position 26 and ends with lysine at position 383 as shown in SEQ ID NO:4 and an anti-CD40 antibody or fragment thereof to the subject before, during and/or after the solid organ or tissue/cellular transplant,

thereby inhibiting rejection of the solid organ or tissue/cellular transplant.

57. (Under Appeal) The method of claim 1 or 9, wherein the alkylating agent is an alkylsulfonate, wherein the alkylsulfonate is busulfan, and wherein the amount of the busulfan that facilitates mixed hematopoietic chimerism is an amount selected from any of 4 mg/kg weight of the subject, 10 mg/kg weight of the subject, 20

mg/kg weight of the subject, 30 mg/kg weight of the subject, between 4-16 mg/kg weight of the subject, or between 0.1 to 20 mg/kg weight of the subject.

58. (Under Appeal) The method of claim 34, 55, or 56, wherein the amount of busulfan that facilitates mixed hematopoietic chimerism is an amount selected from any of 4 mg/kg weight of the subject, 10 mg/kg weight of the subject, 20 mg/kg weight of the subject, 30 mg/kg weight of the subject, between 4-16 mg/kg weight of the subject, or between 0.1 to 20 mg/kg weight of the subject.

59. (Under Appeal) The method of claim 1 or 9, wherein the alkylating agent is an alkylsulfonate, wherein the alkylsulfonate is busulfan, and wherein the amount of the busulfan that facilitates mixed hematopoietic chimerism is an amount below the LD_{50} dose of 136 mg/kg.

60. (Under Appeal) The method of claim 34, 55, or 56, wherein the amount of busulfan that facilitates mixed hematopoietic chimerism is an amount below the LD_{50} dose of 136 mg/kg.

61. (Canceled)

62. (Under Appeal) A method of reducing rejection of a solid organ or tissue/cellular transplant in a subject in need thereof comprising:

a) administering a first dose of T cell depleted bone marrow cells to the subject;

b) administering costimulatory blockade that blocks T cell costimulatory signals in the subject before, during or after the solid organ or tissue/cellular transplant;

c) administering busulfan to the subject in an amount below the LD_{50} dose of 136 mg/kg; and

d) administering a second dose of T cell depleted bone marrow cells to the subject,

thereby reducing rejection of the solid organ or tissue/cellular transplant.

63. (Under Appeal) A method of inhibiting rejection of a solid organ or tissue/cellular transplant in a subject having a transplanted tissue comprising:

a) administering T cell depleted bone marrow cells to the subject;

b) subsequently administering an alkylating agent to the subject in an amount below the LD_{50} dose of 136 mg/kg, wherein the alkylating agent is selected from a group consisting of alkylsulfonates, nitrogen mustards, oxazaposporines, nitrosoureas, and alkylating chemotherapeutic agents;

c) administering a second dose of T cell depleted bone marrow cells to the subject; and

d) administering to the subject costimulatory blockade that blocks T cell costimulatory signals in the subject before, during or after the transplant.

64. (Under Appeal) The method of claim 1, 9, or 63, wherein the alkylsulfonate is selected from a group consisting of busulfan, alkyl p-toluenesulfonates, alkyltrifluoromethanesulfonates, p-bromophenylsulfonates, and alkylarylsulfonates; wherein the nitrogen mustard is selected from a group consisting of mechlorethamines, chlorambucil, melphalan, and uracil mustard; wherein the oxazaposporine is selected from a group consisting of cyclosphosphamide, perfosfamide, and trophosphamide; and wherein the alkylating chemotherapeutic agents is selected from a group consisting of carmustine, cisplatin, lomustine, procarbazine, thiotepa, uracil mustard, triethylenemelamine, pipobroman, streptozocin, ifosfamide, dacarbazine, carboplatin, and hexamethylmelamine.

65. (Under Appeal) The method of claim 48, wherein the soluble CTLA4 mutant molecule is encoded by DNA deposited as ATCC number PTA-2104.

66. (Under Appeal) The method of claim 48, wherein the soluble CTLA4 mutant molecule is expressed by DNA deposited as ATCC number PTA-2104.

67. (Under Appeal) The method of claim 6, 13, or 37, wherein the CTLA4Ig is encoded by DNA deposited as ATCC number 68629.

68. (Under Appeal) The method of claim 6, 13, or 37, wherein the CTLA4Ig is expressed by DNA deposited as ATCC number 68629.

69. (Under Appeal) The method of claim 6, 13, or 37, wherein the CTLA4Ig has the amino acid sequence of a CTLA4Ig fusion protein expressed by a cell deposited as ATCC CRL-10762.

70. (Under Appeal) The method of claim 6, 13, or 37, wherein the CTLA4Ig has the amino acid sequence of a CTLA4Ig fusion protein secreted by a cell deposited as ATCC Accession No. CRL-10762.

71. (Under Appeal) The method of claim 48, wherein the soluble CTLA4 mutant molecule has the amino acid sequence of a soluble CTLA4 mutant molecule expressed by a cell having DNA of SEQ ID NO:3 beginning with guanine at position 76 and ending with adenine at position 1149.

72. (Under Appeal) The method of claim 48, wherein the soluble CTLA4 mutant molecule has the amino acid sequence of a soluble CTLA4 mutant molecule secreted by a cell having DNA of SEQ ID NO:3 beginning with guanine at position 76 and ending with adenine at position 1149.

73. (Under Appeal) The method of claim 6, 13, or 37, wherein the CTLA4Ig has the amino acid sequence of a CTLA4Ig fusion protein expressed by a cell having

DNA of SEQ ID NO:13 beginning with guanine at position 76 and ending with adenine at position 1149.

74. (Under Appeal) The method of claim 6, 13, or 37, wherein the CTLA4Ig has the amino acid sequence of a CTLA4Ig fusion protein secreted by a cell having DNA of SEQ ID NO:13 beginning with guanine at position 76 and ending with adenine at position 1149.

J. EVIDENCE APPENDIX

1. American Type Culture Collection Receipt Issued Pursuant to Rule 7.3 and Viablility Statement Issued Pursuant to Rule 10.2 for ATCC CRL-10762 (pages 43-44)
2. American Type Culture Collection Receipt Issued Pursuant to Rule 7.3 and Viablility Statement Issued Pursuant to Rule 10.2 for ATCC 68629 (pages 45-46)
3. Kean, Leslie S. et al., *Blood*, 1992, 99:1840-9 (pages 47-57)
4. Guo, Zhong et al., *American Journal of Transplantation*, 2003, 3:1091-8 (pages 58-66)
5. Shirasugi, Nozomu et al., *Journal of Immunology*, 2002, 169:2677-84 (pages 67-75)

Evidence Appendix 1

U.S. Serial No. 10/057,288

American Type Culture Collection Receipt Issued Pursuant to Rule 7.3 and Viablility Statement Issued Pursuant to Rule 10.2 for ATCC CRL-10762

American Type Culture Collection

12301 Parklawn Drive • Rockville, MD 20852 USA • Telephone (301)231-5520 Telex: 898-095 ATCCNORTH • FAX: 301-770-2367

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Bristol-Myers Squibb Company
Attention: Peter Linsley
3005 First Avenue
Seattle, WA 98121

Deposited on Behalf of: Bristol-Myers Squibb Company

Identification Reference by Depositor:	ATCC Designation
Escherichia coli MC1061/P3 with plasmid, B7Ig	68627
Escherichia coli MC1061/P3 with plasmid, CD28Ig	68628
Escherichia coli MC1061/P3 with plasmid, CTLA4Ig	68629

The deposits were accompanied by: ___ a scientific description X a proposed taxonomic description indicated above.

The deposits were received May 31, 1991 by this International Depository Authority and have been accepted.

AT YOUR REQUEST:

X We will inform you of requests for the strains for 30 years.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested June 4, 1991. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Bobbie A. Brandon

Bobbie A. Brandon, Head, ATCC Patent Depository ..... Date: June 6, 1991

cc: Brian W. Poor

Evidence Appendix 2

U.S. Serial No. 10/057,288

American Type Culture Collection Receipt Issued Pursuant to Rule 7.3 and Viablility Statement Issued Pursuant to Rule 10.2 for ATCC 68629

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INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3 AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

Bristol-Myers Squibb Company
Attention: Peter Linsley
3005 First Avenue
Seattle, WA 98121

Deposited on Behalf of: Bristol-Myers Squibb Company

Identification Reference by Depositor:	ATCC Designation
Chinese Hamster Ovary Cell Line, CTLA4 Ig-24	CRL 10762

The deposit was accompanied by: ___ a scientific description ___ a proposed taxonomic description indicated above.

The deposit was received May 31, 1991 by this International Depository Authority and has been accepted.

AT YOUR REQUEST:

X We will inform you of requests for the strain for 30 years.

The strain will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strain.

If the culture should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace it with living culture of the same.

The strain will be maintained for a period of at least 30 years after the date of deposit, and for a period of at least five years after the most recent request for a sample. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the culture cited above was tested June 7, 1991. On that date, the culture was viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Bobbie A. Brandon, Head, ATCC Patent Depository — Date: June 10, 1991

cc: Brian W. Poor

Form BP4/9

Evidence Appendix 3

U.S. Serial No. 10/057,288

Kean, Leslie S. et al., *Blood*, 1992, 99:1840-9

A cure for murine sickle cell disease through stable mixed chimerism and tolerance induction after nonmyeloablative conditioning and major histocompatibility complex–mismatched bone marrow transplantation

Leslie S. Kean, Megan M. Durham, Andrew B. Adams, Lewis L. Hsu, Jennifer R. Perry, Dirck Dillehay, Thomas C. Pearson, Edmund K. Waller, Christian P. Larsen, and David R. Archer

The morbidity and mortality associated with sickle cell disease (SCD) is caused by hemolytic anemia, vaso-occlusion, and progressive multiorgan damage. Bone marrow transplantation (BMT) is currently the only curative therapy; however, toxic myeloablative preconditioning and barriers to allotransplantation limit this therapy to children with major SCD complications and HLA-matched donors. In trials of myeloablative BMT designed to yield total marrow replacement with donor stem cells, a subset of patients developed mixed chimerism. Importantly, these patients showed resolution of SCD complications. This implies that less toxic preparative regimens, purposefully yielding mixed chimerism after transplantation, may be sufficient to cure SCD without the risks of myeloablation. To rigorously test this hypothesis, we used a murine model for SCD to investigate whether nonmyeloablative preconditioning coupled with tolerance induction could intentionally create mixed chimerism and a clinical cure. We applied a well-tolerated, nonirradiation-based, allogeneic transplantation protocol using nonmyeloablative preconditioning (low-dose busulfan) and costimulation blockade (CTLA4-Ig and anti-CD40L) to produce mixed chimerism and transplantation tolerance to fully major histocompatibility complex–mismatched donor marrow. Chimeric mice were phenotypically cured of SCD and had normal RBC morphology and hematologic indices (hemoglobin, hematocrit, reticulocyte, and white blood cell counts) without evidence of graft versus host disease. Importantly, they also showed normalization of characteristic spleen and kidney pathology. These experiments demonstrate the ability to produce a phenotypic cure for murine SCD using a nonmyeloablative protocol with fully histocompatibility complex–mismatched donors. They suggest a future treatment strategy for human SCD patients that reduces the toxicity of conventional BMT and expands the use of allotransplantation to non–HLA-matched donors. (Blood. 2002;99:1840-1849)

Introduction

Patients with sickle cell disease (SCD) suffer from both episodic acute complications and chronic, progressive, multisystem decline. Although medical treatments are life-extending, only stem cell transplantation offers an effective cure. There are currently 2 major barriers to stem cell transplantation for SCD: (1) the high morbidity and mortality associated with conventional bone marrow transplantation (BMT) and (2) the scarcity of acceptable stem cell donors.[1,2] (1) Conventional BMT can cure SCD but requires toxic myeloablative preconditioning regimens to achieve donor cell engraftment.[3,4] These intensive preparative regimens have many toxic adverse effects, including potential organ failure and a long-term risk of malignancy. In heavily pretreated patient populations, the morbidity and mortality of transplantation can outweigh the morbidity and mortality of SCD.[2] A dilemma is now developing between early treatment with stem cell transplantation (shown to increase survival and disease-free survival when compared with transplantation after more disease-related complications have occurred) and a delayed approach, during which medical management ameliorates the symptoms of SCD until a later age when definitive therapy can be instituted.[1,2] Unfortunately, this latter course may decrease the chance of successful stem cell transplantation. (2) The paucity of matched related donors has severely limited the number of SCD patients eligible for transplantation. In fact, in the Seattle consortium study, only 6.5% of potential SCD patients were found to be eligible for stem cell transplantation based on disease severity, and of these only 14% had an HLA-matched related donor.[1,3] The lack of matched donors compounds the problem of transplantation-mediated toxicity due to the aggressive regimens used to gain alloengraftment. Thus, for widespread transplantation to be successful, it is essential to develop methods for the generation of allochimerism that have low levels of morbidity and mortality.

The transplantation experience with SCD suggests that a cure can be achieved even without total replacement of recipient stem cells.[3-6] Walters et al reported that 4 of 50 patients treated with

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conventional myeloablative preconditioning unintentionally developed mixed donor/recipient hematopoiesis.[3] Importantly, those patients with stable mixed chimerism developed no further sickle-related complications. Recently, Iannone et al achieved varying degrees of mixed normal/sickle hematopoiesis by titrating bone marrow from a sickle mouse into a lethally irradiated normal mouse.[7] Increasing levels of sickle red blood cells (RBCs) in the chimeric mice resulted in increasing hematopoietic and solid organ pathology. Importantly, solid organ pathology was reported even at sickle RBC percentages that normalized hematologic parameters, such as the reticulocyte count and hemoglobin level. This implies that relying on partial correction of hematologic indices may not predict true systemic cure of SCD in the setting of moderate levels of mixed RBC chimerism. Although the Iannone study was able to investigate certain pathophysiologic sequelae of mixing normal and sickle red cells, many questions about the effect of mixed chimerism could not be addressed due to the recipient mice lacking the sickle genotype.

Given the results of recent studies, there is expanding interest in protocols that are nonmyeloablative and that intentionally produce stable mixed chimerism.[8,9] The problem to overcome is one of tolerance because there must be a coexistence of both host and donor cells for stable mixed chimerism to be achieved. While the initial protocols used relatively nonspecific immunosuppressive agents to induce transplantation tolerance, recent murine studies have focused on blocking T-cell activation pathways as a targeted approach for developing donor-specific tolerance and long-term mixed chimerism.[10-15] These studies have shown that disruption of the T-cell costimulation signal mediated by the CD28/B7 or CD40/CD40L pathways at the time of BMT can lead to anergy of donor-reactive host T cells and produce long-term tolerance to the graft.[10-15] Our group has developed a novel regimen for murine BMT that employs nonmyeloablative preconditioning with low-dose busulfan coupled with costimulation blockade of the CD28/B7 and CD40L pathways.[14] This regimen produces long-term, mixed chimerism and robust tolerance to a fully major histocompatibility complex (MHC)–mismatched allograft without the requirement of myeloablation in a variety of mouse strains.

To rigorously test the ability for such a transplantation regimen to be successful in SCD, we used a transgenic knock-out mouse that lacks all murine hemoglobins and instead produces exclusively human α-globin, γ-globin, and sickle β-globin.[16] The development of this and its related model[17] were landmarks in SCD research because they represent the most authentic genetic representation of human SCD and replicate much of the complex multiorgan disease characteristics present in human SCD patients.[16,17] Although this new murine model of SCD is widely held to be one of the most authentic reproductions of human SCD created to date, there has previously never been a demonstration of BMT and engraftment of donor marrow in this model. Here we show for the first time that nonmyeloablative preconditioning with busulfan coupled with costimulation blockade can safely produce stable white blood cell (WBC) mixed chimerism and total replacement of the peripheral red cell compartment, resulting in a phenotypic cure of murine SCD. Furthermore, this cure is accomplished with fully MHC-mismatched donor marrow. Importantly, the hematologic cure that occurred with total replacement of the red cell compartment was accompanied by normalization of characteristic sickle organ pathology, indicating a total-body amelioration of disease. These results point the way to future trials in human SCD patients, with the goal of creating mixed chimerism across MHC barriers without the prohibitive toxicity of the current standard BMT therapy.

Materials and methods

Animals used in the study

Sickle mice[16] were generously supplied by Dr Paszty at the Lawrence Berkley National Laboratory and are currently maintained at Emory University. Transplant recipients (males, 7-12 weeks) expressing exclusively human α- and sickle β-globin were bred by selective mating and exist on a mixed genetic background (strains: FVB/N, 129, DBA/2, C57BL/6, and Black Swiss). BALB/c and SJL mice (The Jackson Laboratory, Bar Harbor, ME) were used as bone marrow donors. BALB/c and C3H/HeJ mice were used for tests of donor-specific tolerance, and C57BL/6 mice were used as hematologically normal control mice (Jax).

BMT protocol

Recipient mice received 2×10^7 BALB/c or SJL T-cell–depleted bone marrow (TDBM) on day 0. T-cell depletion was accomplished with anti-CD3, anti-CD4, anti-CD8 antibodies (Miltenyi, Auburn, CA). After depletion, T cells represented less than 0.4% of marrow as determined by flow cytometry. Busulfan (20 mg/kg, intraperitoneally; Busulfex, Orphan Medical, Minnetonka, MN) was administered on day −1, and 500 μg each of hamster antimouse CD40L monoclonal antibody (MR1, BioExpress, Lebanon, NH) and human CTLA4-immunoglobulin (CTLA4-Ig) (Bristol-Myers Squibb, Princeton, NJ) (for costimulation blockade) were administered intraperitoneally on days 0, 2, 4, and 6 relative to the BMT. Control mice received 1 of 4 control protocols: (1) busulfan, 20 mg/kg alone, without bone marrow rescue; (2) TDBM alone, without busulfan or costimulation blockade; (3) TDBM plus busulfan, without costimulation blockade; and (4) TDBM plus costimulation blockade, without busulfan. The baseline hematologic parameters were measured 1 week prior to transplantation, and chimerism was tested 2 weeks, 4 weeks, and at monthly intervals after transplantation.

Flow cytometric analysis

Peripheral blood was analyzed by staining with fluorochrome-conjugated antibodies (anti-CD3, anti-CD5, anti-CD11b, anti-GR1, anti-B220, anti–H-2K^d, anti–H-2K^s, anti–H-2K^b, anti-Vβ5.1/5.2, Pharmingen, San Diego, CA; and anti-CD4 and anti-CD8, Caltag Laboratories, Burlingame, CA) or Ig isotype controls (Pharmingen) followed by RBC lysis and washing with a whole blood lysis kit (R&D Systems, Minneapolis, MN). Stained cells were analyzed either using WinList (Verity Software House, Topsham, ME) or CellQuest (Becton Dickinson, Mountain View, CA) software on either a FACScan or FACSCalibur flow cytometer (Becton Dickinson). WBC chimerism was determined by staining with either donor (anti-H2K^d [BALB/c] or anti-H2K^s [SJL]) or recipient (anti-H2K^b) antibodies and specific lineage markers and analysis by flow cytometry. Background staining with anti-H2K^d or anti-H2K^s was typically less than 1%. Vβ deletion was determined by staining with Vβ5 antibodies and specific lineage markers and analysis by flow cytometry.

Analysis of hematologic characteristics

Complete blood counts were performed on a Hemavet 1500 blood analyzer (1500 R series, CDC Technologies, Oxford, CT). Reticulocyte counts were performed by flow cytometry of peripheral blood labeled with antibodies specific for RBCs (anti–Ter-119, Pharmingen) and WBCs (anti-CD45, Pharmingen) and a fluorescent label of RNA, thiazole (Sigma, St Louis, MO). Reticulocyte counts were defined as the percent of peripheral blood cells that were Ter-119$^+$, thiazole$^+$, and CD45$^-$. "Stress" reticulocytes[18] were also analyzed by labeling with an antibody against the transferrin receptor (CD71, Pharmingen).

Blood smears

Smears of peripheral blood were made under ambient air (ie, oxygenated conditions) and then Wright-stained prior to microscopic analysis.

Tissue histology

Tissues were immersion-fixed in 4% paraformaldehyde prior to embedding and cutting. After hematoxylin and eosin staining, a veterinary pathologist examined the sections in a blinded manner.

RBC studies

RBC population half-life. Half-life was determined by a pulsed biotinylation experiment performed essentially as previously described.[19] Briefly, 50 mg/kg N-hydroxysuccinimide biotin (Calbiochem, San Diego, CA) (initially dissolved at a concentration of 50 mg/mL in N,-N,-dimethylacetamide and diluted into 250 μL normal saline just prior to use) was injected intravenously into engrafted or naive sickle animals. This produced a biotin pulse label to the peripheral blood. Blood was obtained either from the retro-orbital venous plexus or through a tail nick at regular intervals after biotinylation. The percentage of peripheral RBCs that were biotinylated was determined by flow cytometry using fluorescent streptavidin-cychrome (Pharmingen) to identify biotinylated cells and a fluorescent Ter-119–phycoerythrin antibody (Pharmingen) to identify RBCs. The decay of biotinylation is directly related to the clearance of the biotinylated RBCs from the peripheral circulation and thus can be used to determine the half-life of the RBC population.

Plasma-membrane phosphatidylserine exposure. Exposure was measured by the percentage of cells that were positive in annexin V (Pharmingen) binding assays. Annexin V binding assays were performed by incubating 1×10^6 peripheral blood cells with 5 μL annexin V and appropriate lineage-specific antibodies in annexin binding buffer (Pharmingen) for 30 minutes at room temperature. Cells were then washed once with annexin binding buffer and analyzed by flow cytometry to determine the percentage of annexin V^+ cells.

Scramblase assays. RBC scramblase enzyme assays were performed essentially as previously described.[20,21] Briefly, 2×10^6 peripheral blood cells were incubated with 3 mM of the fluorescent phosphatidylcholine analog palmitoyl-C_6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidylcholine (NBD-PC; Avanti Polar Lipids, Birmingham, AL) in phosphate-buffered saline containing 1 mM $CaCl_2$ for 30 minutes at 37°C. Cells were then cooled on ice and washed into buffer containing 10 mg/mL defatted bovine serum albumin (Sigma) to back-exchange noninternalized phospholipid. Cells were then incubated with appropriate lineage-specific antibodies (Pharmingen) for 20 minutes at 4°C prior to analysis by flow cytometry.

Analysis of RBC chimerism

RBC chimerism was determined by differential hemoglobin electrophoresis of donor and recipient hemoglobin. Donor β-globin consists of murine "major" and "minor" β-globin isomers, which have different electrophoretic mobilities than recipient human sickle β-globin. Hemoglobin electrophoresis was performed on the Helena Titan III electrophoresis system (Helena Laboratories, Beaumont, TX). Gels were scanned, and percent donor or recipient hemoglobin was determined by densitometry using Kodak 1-D Image Analysis software (Kodak, Rochester, NY).

Determining tolerance to donor antigen by CFSE assays

Splenic and mesenteric lymph node cells were harvested from experimental mice. After RBC lysis and nylon wool passage, cells were incubated in 10 μM of the fluorescent dye, CFSE (Molecular Probes, Eugene, OR). Irradiated (1.8 Gy [1800 rads]) sickle, BALB/c, or C3H mice then received 1×10^7 to 1×10^9 CFSE-labeled cells intravenously. After 66 to 72 hours, splenocytes were harvested from the recipients, the RBCs lysed, and the remaining cells stained with anti-CD4 and anti-CD8 or isotype controls and analyzed by flow cytometry as described above.

Determining hematopoietic balance in recipient hematopoietic organs

Mice were killed, and their splenocytes and bone marrow were harvested with conventional techniques. Hematopoietic balance was specified by determining the percent of bone marrow and spleen cells that were either RBCs (Ter-119^+, $CD45^-$), reticulocytes (Ter-119^+, $CD45^-$, thiazole$^+$), or WBCs (Ter-119^-, $CD45^+$).

Results

Creation of stable chimeras using busulfan and costimulation blockade

Sickle mice[16] were generously provided by Paszty and colleagues and treated with a regimen that included busulfan (20 mg/kg) pretreatment on day −1, BMT with TDBM from BALB/c mice on day 0, and costimulation blockade with 500 μg each of anti-CD40L and CTLA4-Ig on days 0, 2, 4, and 6. The T-cell content of transplanted cells was determined by flow cytometry and represented less than 0.4% of donor cells (data not shown). Additional animals received control protocols, including either (1) 20 mg/kg of busulfan without any bone marrow rescue, (2) TDBM alone, (3) TDBM and busulfan but no costimulation blockade, or (4) TDBM and costimulation blockade but no busulfan.

Figure 1 shows the transient myelosuppressive effect of 20 mg/kg busulfan given to 10 sickle mice. All recipients survived the busulfan treatment and recovered hematopoiesis without the need for stem cell rescue (absolute neutrophil count nadir = 1600 at 14 days, platelet nadir = 170 000 at 14 days). Thus, pretreatment with 20 mg/kg busulfan is nonmyeloablative in the sickle mice.

Mice who received control protocols, including BMT alone (5 mice), BMT after pretreatment with 20 mg/kg busulfan (no costimulation blockade, 5 mice), or BMT and costimulation blockade (no busulfan, 9 mice) showed low to undetectable levels of peripheral WBC chimerism (< 2.5%) throughout the experimental period (Figure 2A). In contrast, 10 of 13 mice who received the full protocol of BMT, 20 mg/kg busulfan, and costimulation blockade achieved multilineage WBC mixed chimerism (Figure 2A). Two of these engrafted mice died during anesthesia at 3

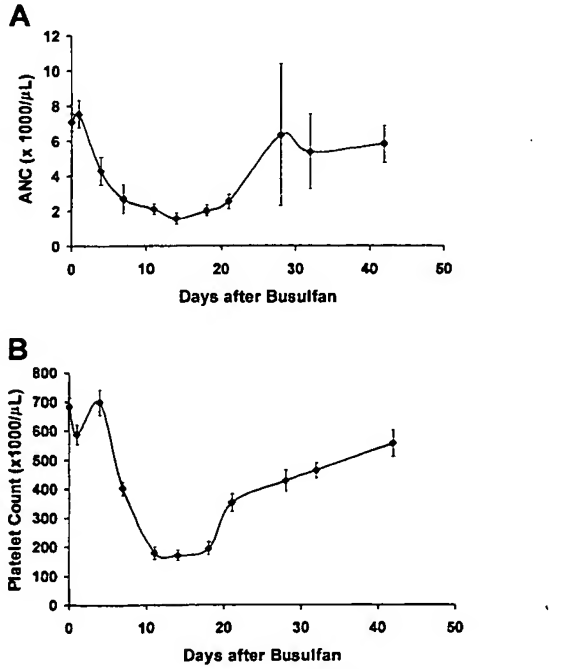

Figure 1. Treatment with 20 mg/kg busulfan is nonmyeloablative in sickle mice. (A) Average absolute neutrophil count (ANC, from automated complete blood count analysis) of 10 sickle mice treated with 20 mg/kg busulfan. (B) Platelet count (from automated complete blood count analysis) of 10 sickle mice treated with 20 mg/kg busulfan.

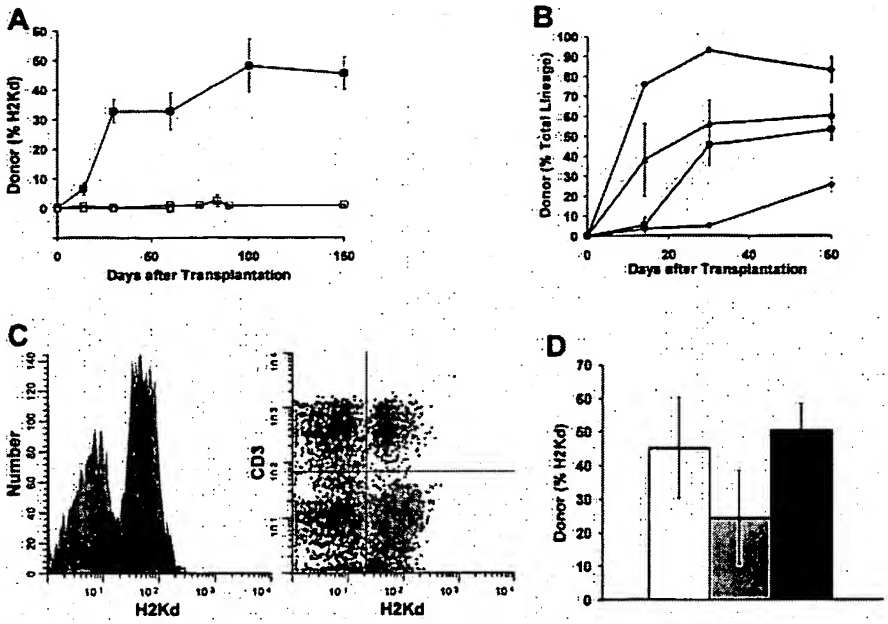

Figure 2. Sickle mice treated with BMT after busulfan and costimulation blockade developed stable WBC mixed chimerism in the peripheral blood and the hematopoietic organs. (A) Mean WBC chimerism in the peripheral blood. Sickle mice (H2-K^b) recipients were treated with 20 mg/kg busulfan (intraperitoneally) on day −1, with 500 μg CTLA4-Ig and anti-CD40L intraperitoneally on days 0, 2, 4, and 6, and underwent transplantation with allogeneic BALB/c (H2-K^d) TDBM (2×10^7 cells intravenously on day 0). The data in this figure represent the mean percent engraftment for 7 engrafted mice that were fully analyzed for more than 150 days (■). Three mice became transiently engrafted but rejected their grafts on or before 60 days. These mice completely reverted to the host WBC and RBC phenotype by 3 to 4 months after transplantation (data not shown). Control animals received 1 of 3 treatments: TDBM alone (n = 5, ○), TDBM plus busulfan (no costimulation blockade, n = 5, △), or TDBM plus costimulation blockade but no busulfan (n = 9, □) Animals in each of the control groups showed less than 2.5% peripheral WBC chimerism. (B) Lineage-specific chimerism over time. The percent of each lineage (granulocytes [GR1], macrophages [CD11b], B cells [B220], and T cells [CD3]) that was donor type (H2-K^d) was identified by flow cytometry over time. Values shown are the mean for each time point from 7 engrafted animals ± SEM. ■ indicates B220; ●, GR1; ▲, CD11b; ◆, CD3. (C) Representative flow cytometric analysis. At left is a histogram showing H2-K^d staining of a chimeric animal with both host (H2-K^d-negative) and donor (H2-K^d-positive) WBCs. At right is a dot plot showing a representative quadrant analysis of CD3 cells versus H2-K^d. (D) WBC chimerism in the bone marrow (■), spleen (□), and thymus (▨). Engrafted sickle cell mice (n = 4) showed 45% ± 15% chimerism (H2-K^d-positive cells by flow cytometry) in the splenic compartment, 50% ± 10% chimerism in the bone marrow, and 25% ± 15% chimerism in the thymus.

months after transplantation (with no signs of graft versus host [GVH] disease prior to their death), and 1 mouse was killed at 2 months for histologic analysis. Chimerism for the remaining 7 mice (mean, 43% ± 10%) peaked 3 months after transplantation and was stable for more than 150 days (Figure 2A). Three mice rejected the graft. These mice displayed transient levels of WBC chimerism at 1 to 2 months after transplantation but fully reverted to the recipient WBC and RBC phenotype by 3 to 4 months after transplantation (data not shown).

Figure 2B shows the engraftment kinetics of individual cell lineages in the peripheral blood of chimeric mice. As expected, donor granulocytes (GR1) and macrophages (CD11b) appear first in the periphery, followed by donor B cells (B220) and T cells (CD3). Figure 2C shows representative flow cytometric data from which the values shown in Figure 2A,B were drawn. The peripheral chimerism in individual engrafted animals was reflected in their hematopoietic organs. Mean chimerism for bone marrow (50%), spleen (45%), and thymus (25%) is shown in Figure 2D.

The chimerism noted in the animals that received the full protocol of BMT, 20 mg/kg busulfan, and costimulation blockade was not donor strain–specific, because 5 sickle mice undergoing transplantation with busulfan, costimulation blockade, and TDBM from SJL mice (which are of the H-$2K^s$ rather than H-$2K^d$ genotype) showed similar levels of WBC and RBC chimerism (data not shown). Engrafted mice showed no signs of GVH disease: Their individual body weights remained stable (mean pretransplantation weight, 32 ± 1 g; mean posttransplantation weight, 36 ± 4 g) and posttransplantation necropsies, in which organs (including the skin, heart, lungs, spleen, liver, kidney, and intestinal tract) were specifically evaluated for signs of GVH disease, showed normal histology.

Peripheral white cell chimerism was comparable to previous results using busulfan and costimulation blockade in allogeneic wild-type mice,[14] whereas peripheral RBC chimerism was strikingly higher in the sickle transplantation recipients. Quantification of donor (normal BALB/c β-globin, major and minor alleles) and recipient (human sickle β-globin) hemoglobins separated by cellulose acetate electrophoresis showed 78% to 90% donor chimerism within 2 weeks that reached 100% by 1 month in all of the engrafted sickle mice (Figure 3). The complete replacement of host hemoglobin was stable for the entire experimental duration (> 150 days after transplantation). The higher level of peripheral erythroid chimerism compared with WBC chimerism is consistent with the

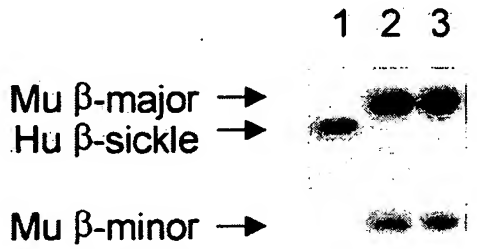

Figure 3. Stable, complete replacement of the peripheral RBC compartment occurred in engrafted mice. RBC chimerism is determined by hemoglobin electrophoresis. Recipient mice originally possessed only human sickle β-globin (lane 1), while donor mice possessed the major and minor alleles of mouse β-globin (lane 2). Lane 3 shows a representative engrafted mouse with complete peripheral replacement with donor β-globin. Complete replacement of the peripheral blood with donor hemoglobin occurred within 1 month after transplantation and was stable for the entire observation period (> 150 days) in all engrafted mice. Hu indicates human; Mu, murine.

accumulation and enhanced survival of normal RBCs in an environment with rapid turnover and degradation of sickle RBCs.

Mice receiving TDBM alone or TDBM plus busulfan (no costimulation blockade) showed no donor RBC (hemoglobin electrophoresis) or WBC (flow cytometry) engraftment. Thus, in the absence of costimulation blockade, the sickle mice were able to totally reject the donor graft. However, in the setting of BMT plus costimulation blockade (but no busulfan), 5 of 9 control mice developed significant red cell chimerism (10%-54%, Figure 4) despite minimal WBC chimerism (< 2.5%, Figure 2A). This probably reflects very low levels of stem cell engraftment and massive expansion of healthy red cell precursors, leading to significant peripheral read-out of donor hemoglobin.

Chimeric mice are specifically tolerant to donor and recipient antigen

BALB/c mice express I-E and therefore delete Vβ5-bearing T cells, whereas sickle mice do not express I-E and specifically utilize Vβ5.1/2 on about 2% to 3% of $CD4^+$ T cells.[22,23] As anticipated, the 9 animals that received BMT and costimulation blockade but no busulfan (and who showed low to undetectable WBC engraftment, Figure 2A) failed to delete donor-reactive $V\beta5^+CD4^+$ T cells (Figure 5A). In contrast, engrafted animals that received TDBM along with busulfan and costimulation blockade developed near complete deletion of $CD4^+V\beta5^+$ T cells by day 60. Part of this decrease in total $V\beta5^+CD4^+$ T cells could have been due to the mixture of donor ($V\beta5^-$) and recipient ($V\beta5^+$) $CD4^+$ T cells even without deletion. However, given that 82% of $CD4^+$ T cells showed Vβ5 deletion at 150 days (Figure 5A), while $CD4^+$–T-cell chimerism was only 33% ± 8% at this same time point (data not shown), most of the reduction of $V\beta5^+CD4^+$ T cells is likely due to specific $V\beta5^+$–T-cell deletion. The percentage of Vβ8-bearing $CD4^+$ T cells, normally expressed on 15% to 25% of BALB/c and sickle $CD4^+$ T cells, was similar in all groups, indicating that the T-cell deletion was donor-specific in nature (data not shown). These results suggest that the bone marrow–derived I-E–bearing donor cells influence the selection of the T-cell repertoire in engrafted mice, ultimately conferring robust long-term donor-specific tolerance.

Coincident with the long-term chimerism seen in the busulfan-treated animals was the development of specific tolerance to the allogeneic BALB/c bone marrow graft without GVH disease (Figure 5B). A rigorous in vivo alloproliferation assay using CFSE dye was performed to test for the presence of alloreactivity.[24] T cells were harvested from spleens and mesenteric lymph nodes of animals that were at least 100 days after transplantation. After labeling with 10 μM CFSE, T cells were transferred into recipient mice (BALB/c, sickle, or C3H) previously supralethally irradiated. Splenocytes were harvested 72 hours later and analyzed via flow cytometry. While $CD4^+$ and $CD8^+$ T cells from nonengrafted groups underwent extensive cell division in response to both BALB/c and third-party (C3H) hosts but not to sickle hosts (data not shown), T cells from the engrafted mice generated no proliferative response to either donor (BALB/c) or recipient (sickle) hosts (Figure 5B). As expected, clear proliferative responses were present when naive BALB/c T cells were transferred into sickle hosts (data not shown) or when chimeric T cells were transferred to a third party (C3H, Figure 5B). These results confirm the specific absence of donor and recipient alloreactive $CD4^+$ or $CD8^+$ T cells capable of cell division in chimeric animals.

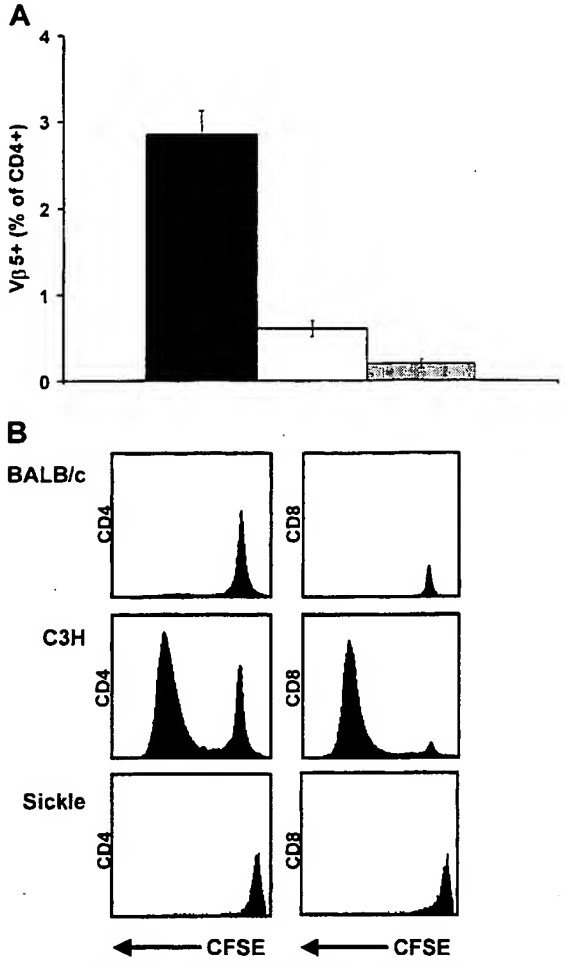

Figure 5. Engrafted mice showed specific tolerance to donor T cells. (A) $CD4^+$ T cells from engrafted animals (□) ceased to utilize Vβ5.1/2, similarly to the donor (BALB/c, ▨). Nonengrafted mice (■) utilized Vβ5.1/2 throughout the observation period. Data show the average of 7 representative mice from each group at 150 days after transplantation. (B) T cells were examined for their proliferative capacity against donor, recipient, and third party using an in vivo alloproliferation model with CFSE-labeled T cells from engrafted and nonengrafted animals. The concentration of CFSE within the cell decreases by 50% after each division. Labeled T cells from engrafted and nonengrafted animals were adoptively transferred into lethally irradiated (1.8 Gy [1800 rads]) BALB/c (donor), sickle (recipient), and C3H (third-party) mice. Histograms of representative animals demonstrate that $CD4^+$ and $CD8^+$ T cells from engrafted recipients are essentially unresponsive to BALB/c (donor) and sickle (recipient) hosts. Both subsets of T cells, however, when transferred into C3H mice (third party) undergo maximal division (up to 8 divisions). Tolerant animals, therefore, show no proliferation to donor or recipient but a normal proliferative response to third party (C3H, $H\text{-}2^k$).

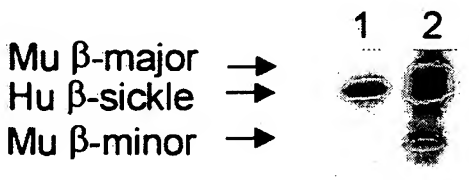

Figure 4. A subset of 5 of 9 mice who received only costimulation blockade (no busulfan) developed significant RBC chimerism. Lane 1 shows a representative mouse without engraftment, with only human β-sickle hemoglobin. Lane 2 shows a representative mouse 3 months after transplantation with RBC chimerism, having both recipient (human β-sickle hemoglobin) and donor (mouse major and minor β-hemoglobin alleles) RBCs. Hu indicates human; Mu, murine.

Chimeric mice are cured of sickle cell disease

Engrafted sickle mice demonstrated a phenotypic cure of their SCD by a variety of parameters. As seen in Figure 6A,B, a striking

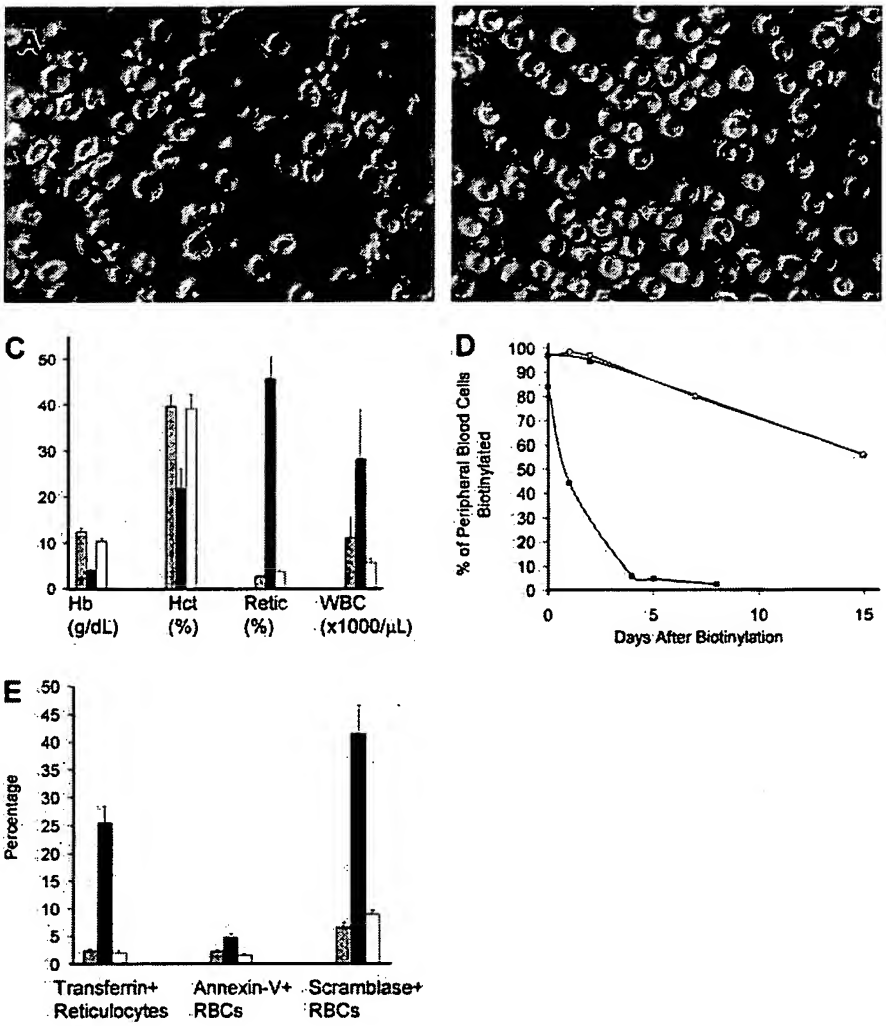

Figure 6. Engrafted mice demonstrated a phenotypic cure of SCD. Peripheral blood smear from representative untreated (A) and engrafted (B) animals, prepared under ambient air. Arrows point to representative irreversibly sickled cells in the untreated blood. Engrafted animals have a normal peripheral smear. Original magnification, × 250. (C) Hematologic parameters are normalized in engrafted mice. Normalization of hematologic parameters was present within 1 month after transplantation and was stable for the entire experimental period (> 150 days). Shown are the mean ± SEM for C57BL/6 controls (n = 6, ▨), nonengrafted mice (n = 4, ■), and engrafted mice (n = 7, □) in a representative experiment performed 3 months after transplantation. Hb indicates hemoglobin; Hct, hematocrit; Retic, thiazole$^-$ reticulocyte percent; WBC, white blood cell count. (D) Engrafted mice have a normal RBC half-life. Peripheral blood was biotinylated at time 0, and the decay of biotinylation in RBCs (identified as Ter-119$^+$, CD45$^-$, biotinylated cells) was monitored by flow cytometry. Untreated sickle animals (■) had an exceedingly short RBC half-life, while engrafted animals (□) had an RBC half-life that was indistinguishable from normal C57BL/6 controls (▲). These data represent the average of 2 animals that were biotinylated 3 months after transplantation. (E) The engrafted RBC population is healthy as determined by normalization of the percentage of transferrin$^+$ stress reticulocytes, PS exposure as measured by annexin V binding, and scramblase activity as measured by NBD-PC internalization. C57BL/6 control (▨, n = 6), nonengrafted (■, n = 4), and engrafted (□, n = 7) animals were analyzed. Shown are the mean ± SEM of animals analyzed 3 months after transplantation. Normalization of RBC parameters in engrafted animals occurred within 1 month after transplantation and was stable for the entire observation period (> 150 days).

absence of irreversibly sickled cells in peripheral blood smears occurred after transplantation in mice that were conditioned with busulfan and costimulation blockade. Engrafted mice also demonstrated normalization of their hematologic abnormalities (Figure 6C), including hemoglobin (45 g/L corrected to 100 g/L [4.5 g/dL corrected to 10 g/dL]), hematocrit (0.16 corrected to 0.40 [16% corrected to 40%]), and peripheral thiazole$^+$ reticulocyte percentage (49% corrected to 3.5%), consistent with a reversal of their hemolytic anemia. Furthermore, the abnormally elevated WBC count seen in naive sickle mice was corrected in engrafted mice (20×10^9/L to $5.1/ \times 10^9$/L [20 000/μL to 5100/μL]).

The health of the newly emerging chimeric red cells was assessed by 3 physiologic markers. First, RBC population half-life was determined through a pulsed biotinylation experiment[19] (Figure 6D). Untreated and engrafted sickle mice were injected intravenously with N-hydroxysuccinimide biotin to label the peripheral blood with biotin as previously described.[19] RBC half-life was determined by the decay of the biotinylated RBCs over time by flow cytometry. Consistent with previous work from this laboratory,[25] RBCs from the naive sickle animals had exceedingly short peripheral half-lives (0.8 days) compared with normal control C57BL/6 mice (half-life 18 days). Engrafted animals had a RBC half-life indistinguishable from that of normal mice, consistent with replacement of the diseased red cell compartment with normal RBCs. Second, we measured the production of transferrin$^+$ "stress" reticulocytes in engrafted and nonengrafted mice. These cells are an indication of overactive erythropoiesis and are thought to contribute to the increased adhesion of sickle reticulocytes in the microvasculature.[18,26,27] Figure 6E shows that the percent of these cells decreases from 27% to 3% in engrafted mice, consistent with normalization of red cell turnover in these animals. Third, we examined plasma membrane phosphatidylserine (PS) exposure, which is known to be increased in sickle red cells.[28,29] PS is thought to contribute to increased clearance of these cells by macrophages and monocytes and may also contribute to abnormal endothelial adhesion.[30] Two assays were used: annexin V binding, which measures exposed PS residues directly,[31] and NBD-PC internalization, which measures the scramblase enzyme that leads to PS exposure on the plasma membrane.[20,21,32] Figure 6E shows that sickle mice consistently show a high PS exposure prior to transplantation (measured by annexin V binding), but engrafted mice demonstrate a significant decrease in this PS exposure. Figure 6E also shows that a dramatic decrease in the number of RBCs with active scramblase occurs after engraftment, consistent with the decline in PS exposure described above.

Although the focus of this study is the effect of full RBC replacement in the face of partial WBC engraftment, as noted above, a subset of mice (5 of 9) receiving BMT and costimulation blockade (but not busulfan) did develop partial RBC chimerism. Of those 5 mice, 2 died unexpectedly (1 at 3 months, 1 at 4 months of age, neither with signs of GVH disease) and thus were not fully analyzed. The remaining mice were analyzed for the effects of

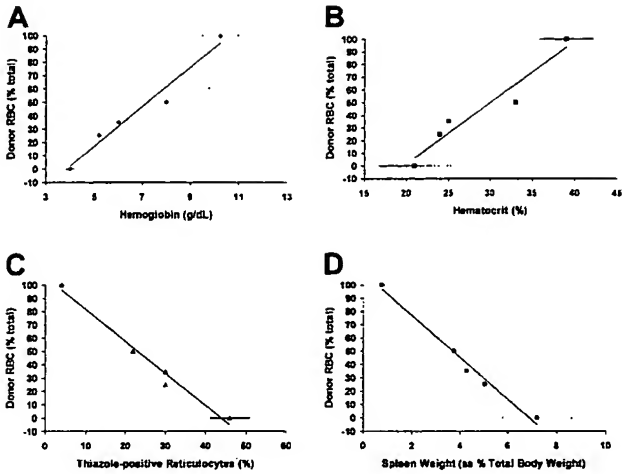

Figure 7. Hematologic parameters are partially normalized in mice with partial RBC chimerism. (A) Hemoglobin concentration (g/dL, as determined by automated complete blood count analysis) versus percent peripheral RBC engraftment. (B) Hematocrit (percentage, as determined by automated complete blood count analysis) versus percent peripheral RBC engraftment. (C) Reticulocyte count (percent thiazole$^+$, Ter-119$^+$ cells determined by flow cytometry) versus percent peripheral RBC engraftment. (D) Spleen size (as percentage of total body weight) versus percent peripheral RBC engraftment. For all panels, age-matched, unengrafted sickle mice were used for 0% engraftment controls (n = 4), and engrafted animals (n = 4) were used as 100% engrafted controls. Each of the other points represents individual mice with varying levels of peripheral RBC engraftment.

mixed RBC chimerism on murine SCD. As shown in Figure 7, there was a progressive normalization of both hematologic (hemoglobin and hematocrit) and physiologic (reticulocyte count and spleen size) parameters in mice with increasing RBC engraftment. Further analysis also showed partial correction of NBD-PC internalization, annexin V binding, and splenic hematopoeitic balance in mice with mixed donor and host RBCs (data not shown). A separate study designed to rigorously determine the effect of progressively increasing amounts of normal RBCs in the sickle background is currently underway (L.S.K. et al, in preparation).

The spleens in the engrafted mice exhibit signs of reversal of characteristic sickle pathology

One of the hallmarks of murine sickle cell pathophysiology is the dramatic increase in spleen size compared with that of normal animals.[16,17,25] This is related to the immense requirement for erythropoiesis in order to replenish the rapid destruction of peripheral sickle RBCs.[16,17,25] As shown in Figure 8A, the spleen undergoes a significant decrease in size in engrafted mice (from 7.3% total body weight in age-matched naive sickle mice to 0.7% total body weight in engrafted mice measured 5 months after transplantation). Figure 8B shows that while the spleen functions as a largely erythropoietic organ in untreated sickle mice, it undergoes a reprogramming in the engrafted cohort and resumes a more normal balance between white and red cell hematopoiesis. Figure 9A,B shows a histologic comparison of the spleens from naive and engrafted mice. In engrafted mice there is a striking resolution of the characteristic hyperactive hematopoiesis and red cell pooling characteristic of SCD and the acquisition of normal splenic architecture.

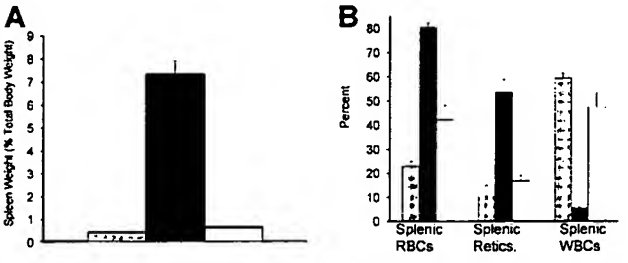

Figure 8. The spleen demonstrated reversal of pathophysiology in engrafted animals. (A) Spleen weight expressed as percent total body weight (mean ± SEM) in C57BL/6 control (▨, n = 5), untreated sickle (■, n = 7), and engrafted (□, n = 4) animals. Spleen weights in engrafted animals were measured 5 months after transplantation. Spleen weights for control mice were age-matched to the engrafted animals. (B) The balance of hematopoiesis in the spleen was also normalized in engrafted mice. The percent RBCs, reticulocytes, and WBCs (mean ± SEM) all approach C57BL/6 control values (▨, n = 5) in engrafted mice (□, n = 4), while untreated sickle animals (■, n = 7) are highly abnormal. Splenic hematopoiesis in engrafted animals was measured 5 months after transplantation. Control mice were age-matched to the engrafted animals.

Renal histology is normal in engrafted mice

In addition to the defects observed in both the peripheral blood and the hematopoietic organs, sickle mice also demonstrate solid organ pathology similar to that seen in patients with SCD. [16,17] As in the original description of this murine SCD model,[16,17] we have noted pathologic changes in many organs, including the kidney, liver, lung, and heart in untreated sickle animals. To determine the effect of BMT on organ structure and histology, necropsies were performed on age-matched naive and engrafted animals and tissues

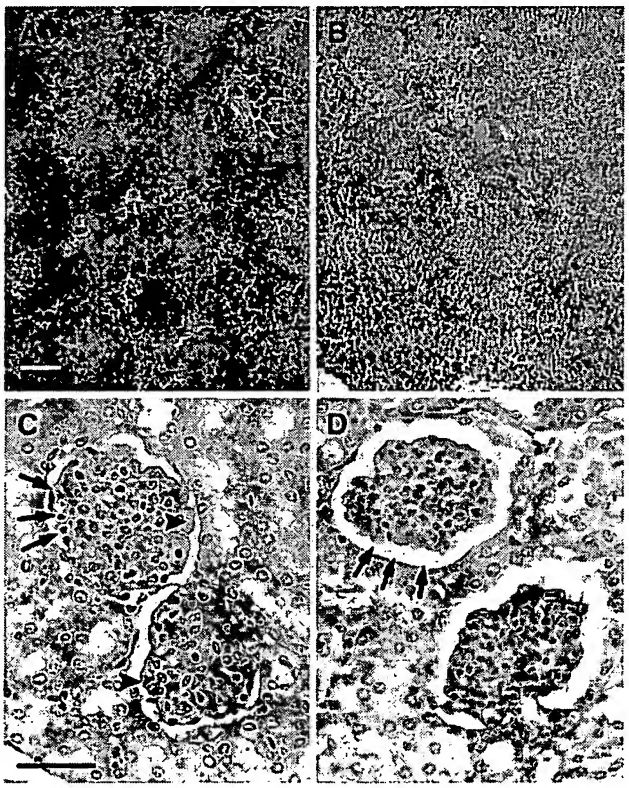

Figure 9. Engrafted mice showed no evidence of characteristic sickle splenic and renal pathology. (A,B) Histology of the spleen in untreated and engrafted mice. (A) Untreated sickle spleen. Splenic architecture is highly abnormal with pooling of sickled RBCs and areas of increased hematopoiesis. (B) Spleen from an engrafted mouse killed 5 months after transplantation. Splenic architecture is now normal. No RBC pooling is present. (C,D) Histology of the kidney in untreated and engrafted mice. (C) Untreated sickle kidney. The sickle kidney shows evidence of membranoproliferative glomerulonephritis. Arrowheads point to thickened glomerular membrane. Arrows point to narrowed glomerular space. (C) Kidney from an engrafted mouse killed 5 months after transplantation. Glomerular architecture is now normal. Sickle mice were age-matched to engrafted mice for this analysis. Sections were stained with hematoxylin and eosin. Bars = 50 μm.

were prepared for histologic analysis. We found that engrafted animals had normal histology of all organs tested, including the kidney, liver, heart, and lungs. Representative of the histologic normalization that occurred in these animals, Figure 9C,D shows a comparison of renal histology in age-matched untreated and engrafted mice. Figure 9C shows the membranoproliferative glomerulonephritis consistently observed in untreated sickle mice. Figure 9D shows that engrafted animals had normal renal histology, including normalization of glomerular capsular space and glomerular membrane thickness.

Discussion

Stem cell transplantation is the only current treatment that offers an effective cure for SCD.[3,4,33,34] However, the availability of this treatment is limited by the lack of HLA-matched related donors and the toxicity of the myeloablative preconditioning regimens.[1,2] While alternative stem cell sources are being explored, such as unrelated cord blood stem cells and partially matched relatives, the use of these types of donor cells has required strong, toxic, preparative regimens to myeloablate all residual host cells. High induction-related morbidity/mortality is not acceptable in SCD, because good medical support can extend life to 40 to 60 years without transplantation.[2] Indeed, the first 2 adults undergoing transplantation by the Seattle consortium died of toxicity-related complications, which halted the adult clinical trial. Moreover, Vermylen et al showed that survival and disease-free survival after BMT were consistently worse in those children who met the Seattle criteria for severe disease than in those who were relatively asymptomatic.[4] BMT will be available to most SCD patients only when a minimally toxic regimen can be established. Furthermore, given the scarcity of matched-related donors, a truly successful approach will need to cross allogeneic barriers.

Walters et al reported that a subset of patients who were treated with a myeloablative preparative regimen and BMT (designed to produce total replacement with donor cells) developed stable mixed chimerism.[3] These mixed-chimeric patients had no further sickle cell–related clinical events. This clinical experience leads to 2 major lines of experimental inquiry: (1) Can a regimen be developed to intentionally produce tolerance and mixed WBC chimerism, and will this result in a reliable cure of SCD? (2) What is the minimal level of RBC chimerism that can lead to both a hematologic and systemic cure of SCD? The first step toward the ultimate goal of answering these questions in a clinical setting is to perform controlled studies in a bona fide animal model of SCD. The recent creation of transgenic mice in which human sickle β-globin exists in a background of normal human α-globin and a complete knock-out of the endogenous murine α- and β-globin genes represent the latest advances toward such a model.[16,17,35] These mice recapitulate much of the hematologic pathology seen in human SCD, including hemolytic anemia (with resulting low hemoglobin concentration, low hematocrit, and high reticulocyte count) and high WBC count. Their RBCs do differ from human sickle RBCs in that they have a lower mean corpuscular hemoglobin concentration, likely resulting from a mild α/β chain imbalance, and subsequent mild thalassemic component to their anemia.[16,17,35] It is speculated that this slight α/β chain imbalance is just enough to prevent this model from being lethal[35] and that the overall authenticity of this model of SCD is not seriously impaired by this difference.[16,17] Importantly, these knock-out models of SCD also show total-body pathology reminiscent of human SCD, including splenic sequestration and enhanced splenic erythropoiesis, renal cortical and glomerular pathology, and liver infarction.[16,17] The creation of this latest generation of murine SCD models thus allows investigation of both the hematologic consequences of mixed hematopoietic chimerism and the effect that engraftment has on organ pathology and the systemic disease.

In this study we have focused on the first question posed above and have intentionally induced mixed WBC chimerism in the transgenic sickle mice with a well-tolerated, nonmyeloablative preconditioning regimen employing low-dose busulfan. Furthermore, we have added costimulation blockade of the CD40/CD40L and CD28/B7 pathways to induce stable donor-specific tolerance to fully MHC-mismatched bone marrow without GVH disease. This protocol resulted in WBC mixed chimerism and complete replacement of the peripheral RBC compartment in 10 of 13 animals within 1 month of transplantation that was stable throughout the observation period (> 150 days). The fact that engrafted mice demonstrated a significantly higher level of donor RBCs in the peripheral blood compared with donor WBCs (100% vs 43%, respectively) is likely due to the dramatically increased clearance of sickle RBCs from the circulation, with RBC read-out reflecting the lineage-specific survival of donor RBCs. Thus, even with partial stem cell engraftment and mixed WBC chimerism, complete normalization of the peripheral RBC defect occurred in engrafted sickle mice.

The replacement of the RBC compartment with normal cells resulted in a clinical cure of murine SCD by all parameters tested, including reversal of hemolytic anemia, disappearance of sickled forms on peripheral blood smears, reduction of RBC PS exposure, and normalization of the hematopoietic balance in the spleen. Furthermore, engrafted mice showed a reduction in peripheral WBC count, which is elevated in the untreated sickle cohort. This reduction in peripheral WBC count is particularly interesting given that several lines of evidence indicate that leukocytes play a major role in sickle cell pathophysiology.[36,37] Indeed, elevated WBC count is an epidemiologic risk factor for increased morbidity and mortality in SCD,[38] including pulmonary complications,[39] stroke,[40] and silent brain infarction.[41] Moreover, decline in WBC count was the hematologic parameter most strongly correlated with clinical response to hydroxyurea in reducing the severity of SCD.[42] Importantly, in addition to the correction of the sickle hematologic abnormalities noted above, engrafted mice also showed normalization of solid organ structure and histology. Again, this is a strong indication that whole-body cure of murine SCD was accomplished with our transplantation protocol.

All sickle mice treated with TDBM alone or TDBM plus busulfan (no costimulation blockade) fully rejected their bone marrow graft and developed no WBC or RBC chimerism. In contrast, significant peripheral RBC chimerism (10%-54%) was obtained in a subset of mice (5 of 9 animals) that did not receive busulfan, but that did receive TDBM and costimulation blockade, despite minimal WBC chimerism (< 2.5% by flow cytometry). The higher level of donor RBCs compared with WBCs in the peripheral blood again likely reflects their survival advantage over the rapidly cleared sickle cells. Given the recent discovery of common lymphoid and myeloid precursors,[43,44] the intriguing possibility also exists of a common erythroid/megakaryocyte precursor and that enhanced engraftment of such a cell may occur in sickle mice. Clinically, the ability to engraft RBCs without any busulfan treatment may be very important. Given the extreme fragility of adult SCD patients subjected to conventional BMT, a

regimen that would produce RBC chimerism without any chemotherapy may allow even the sickest patients an option of long-term disease amelioration. This development of mixed RBC chimerism in a subset of mice given BMT and costimulation blockade without busulfan pretreatment applies to the second question posed above: What is the minimal level of RBC chimerism that can lead to both a hematologic and systemic cure of SCD? Although beyond the scope of the present study, the small number of animals who serendipitously developed mixed RBC chimerism (Figure 7) does give us an indication that progressive hematologic and physiologic cure can result with increasing levels of normal RBCs. This is to be expected given the vast clinical experience with acute and chronic transfusion programs. It is also in agreement with the study by Iannone et al, wherein normal mice underwent transplantation with sickle bone marrow in such a way as to produce progressively increasing levels of sickle RBCs.[7] In that study, mice with increasing levels of sickle RBCs had progressively more abnormal hematologic and organ pathology. We are currently modifying our transplantation regimen to consistently produce varying levels of mixed donor/recipient RBC chimerism in the sickle background (L.S.K. et al, in preparation). Long-term studies with large numbers of mice will be required to produce insights into the total-body effect of mixed RBC chimerism and may indicate whether less than total RBC replacement is a reasonable goal for clinical cure.

Our results demonstrate that a phenotypic cure for murine SCD can be achieved without the significant risks associated with myeloablative BMT regimens. In the murine SCD model, BMT after nonmyeloablative conditioning and tolerance induction leads to stable, robust mixed WBC chimerism across allogeneic barriers. In this study, complete replacement of abnormal RBCs was accomplished in the setting of mixed WBC chimerism, and this complete replacement with donor RBCs led to hematologic and systemic cure of murine SCD. Many questions still exist: Is complete replacement of sickle RBCs required for a full systemic cure, or is there a level of mixed RBC chimerism in which sickle hematologic and organ pathology is consistently and stably corrected? What are the factors that mediate acceptance or rejection of the bone marrow graft in the setting of tolerance induction and nonmyeloablative conditioning? Is the inflammatory state that exists in the background of both sickle mice and sickle patients a contributor to graft rejection? Perhaps most importantly, how will these results be translated to affect human SCD patients? Clearly, this is just the first step in the long road toward developing nonmyeloablative transplantation and tolerance induction for SCD patients. However, although allogeneic barriers are much more significant in man than in mouse, our successful use of costimulation blockade to cross allogeneic barriers suggests that in the future related techniques may be clinically feasible for human SCD and other nonmalignant hematologic disorders.

Acknowledgments

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Evidence Appendix 4

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Long-Term Survival of Intestinal Allografts Induced by Costimulation Blockade, Busulfan and Donor Bone Marrow Infusion

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Tolerance-inducing strategies that infuse donor bone marrow cells in conjunction with costimulation blockade have not been applied to intestinal transplantation. Intestines from BALB/c mice were transplanted into C57BL/6 recipients treated with anti-CD40L mAb, CTLA4-Ig, donor bone marrow, and busulfan. The majority of mice transplanted after completion of this regimen developed hematopoietic macrochimerism, although the degree of chimerism varied widely between recipients, and experienced long-term allograft survival. T cells from these mice demonstrated donor-specific hyporesponsiveness *in vitro*. However, T cells from chimeric mice proliferated to donor alloantigen *in vivo*. Furthermore, chimeric mice bearing intestinal allografts were capable of rejecting subsequently placed donor-strain skin grafts. These data suggest that although long-term allograft survival occurs in the absence of acute or chronic rejection, recipient mice are not completely unresponsive to donor alloantigens. When intestinal transplantation was performed at the time of initial bone marrow infusion (initiation of the chimerism protocol), most recipients failed to develop chimerism and promptly rejected the intestinal allograft. Although this is the most effective protocol that we have tested using this stringent model of transplantation, our observations suggest that modifications will be necessary before it can be reliably applied to the transplantation of highly immunogeneic organs like the intestine.

Key words: Bone marrow transplantation, chimerism, costimulatory blockade, intestinal transplantation

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Introduction

Intestinal transplantation is now recognized as definitive therapy for selected patients with intestinal failure. Despite the increased number of intestinal transplants performed, the outcome of intestinal transplantation remains inferior to that associated with the transplantation of other organs (1). This is mainly because of the greater immunogenicity of intestinal allografts and the associated increased frequency and severity of rejection (2,3). In addition to the problem of rejection itself, the relatively greater amount of immunosuppression required to prevent or control rejection following intestinal transplantation results in significantly increased rates of infection and post transplant lymphoproliferative disease (PTLD) relative to other transplanted organs (4–6).

The broader application of intestinal transplantation awaits the development of more effective and less toxic immunosuppressive regimens. In this regard, immunosuppressive strategies that promote donor-specific tolerance may offer particular benefits to patients undergoing intestinal transplantation. We have previously shown that agents including anti-CD4 mAb, anti-CD40L mAb, anti-LFA1, anti-B7.1 and anti-B7.2 mAb, and CTLA4Ig that promote tolerance and/or long-term survival of allografts in other transplant models fail to produce the same effect in the murine model of intestinal transplantation (7–9) (K. A. Newell, unpublished observations). Of the currently available approaches to tolerance induction, combined donor bone marrow and organ transplantation affords the advantage of inducing a robust tolerance to allografts in a number of experimental models. It was first recognized that hematopoietic chimerism was associated with donor-specific tolerance five decades ago (10,11). In 1955 Main and Prehn demonstrated that donor-specific tolerance could be acquired by infusing bone marrow cells into lethally irradiated adults (12). Concerns about infectious complications in fully allogeneic chimeras prompted investigators to design strategies for inducing mixed allogeneic chimerism (13). Subsequently protocols were designed that replaced the need for lethal irradiation with conditioning regimens that utilized anti-T-cell antibodies, low-dose TBI, and thymic irradiation (14) or costimulatory blockade combined with infusion of very large doses of donor bone marrow (200×10^6 BM cells/mouse) (15,16). However, persisting concerns about the long-term consequences of irradiating transplant recipients and the difficulty of

Z. Guo and J. Wang contributed equally to this work.

obtaining the number of bone marrow cells required for the 'mega-dose' protocols may limit the clinical application of these approaches. Recently we have reported a regimen that completely replaces radiation as a conditioning agent with the alkylating agent busulfan. Busulfan preferentially depletes early hematopoietic stem cells without significantly affecting the number of mature circulating leukocytes in murine transplant models (17). Mice treated with busulfan, anti-CD40L mAb, CTLA4-Ig, and infused with two doses of donor bone marrow developed donor-specific tolerance as indicated by the indefinite survival of primary skin and heart allografts and acceptance of subsequently placed donor, but not third party skin grafts (17,18).

The aim of the current study was to determine the effect of this tolerance-inducing strategy on the outcome of intestinal transplants in mice. The results of our studies demonstrate that the infusion of donor bone marrow together with busulfan and costimulation blockade induces hematopoietic chimerism and promotes the long-term survival of intestinal allografts transplanted into mice that have completed the treatment regimen. This long-term survival is associated with donor-specific hyporesponsiveness *in vitro* and deletion of donor-reactive T cells *in vivo*. Interestingly, mice bearing long-term surviving intestinal allografts displayed significant prolongation of subsequently placed donor-strain skin grafts but were not tolerant by the strictest definition in that most donor-strain skin grafts were eventually rejected. Finally, unlike results obtained using skin and heart transplant models, most intestinal allografts placed on the initial day that the tolerizing regimen was begun were promptly rejected. These results provide a cautionary note and demonstrate that in its current form this approach to tolerance induction may not be clinically applicable for highly immunogenic organ allografts.

Materials and Methods

Mice

Adult male C57BL/6 (H-2^b), BALB/c (H-2^d), C3H/HeJ (H-2^k), and CB6F1/J (C57BL/6 × BALB/c, H-2^{bxd}) mice 6–8 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME) and the National Cancer Institute (Frederick, MD). All mice were housed in specific pathogen-free conditions and in accordance with institutional guidelines. All studies were approved by and performed in compliance with the policies of the Institutional Animal Care and Use Committee of Emory University.

BM preparation and treatment regimens

Bone marrow was flushed from the tibiae, femurs, and humeri of BALB/c mice using sterile saline, needles, and syringes. Single-cell suspensions of harvested bone marrow were made. Red blood cells were lysed using a Trizma base ammonium chloride solution (Sigma, St. Louis, MO). The BM cells were resuspended at 2×10^7 cells/500 μL sterile saline and injected intravenously on days 0 and 6. Hamster antimouse CD40L mAb (MR1; Bioexpress, Lebanon, NH) and CTLA4-Ig (Bristol-Myers Squibb, Princeton, NJ) were administered on days 0, 2, 4, 6 (500 μg/dose i.p). A single 600 μg dose of busulfan (Orphan Medical Inc., Minnetonka, MN) was administered intraperitoneally on day 5.

Transplantation and histologic graft assessment

Intestinal transplantation was performed as described (7). Intestinal grafts were revascularized by anastomosing the portal vein to the recipient inferior vena cava and the superior mesenteric artery to the recipient infrarenal aorta. The jejunum was exteriorized as a stoma and the ileum was anastomosed to the side of the recipient jejunum. Recipient mice were sacrificed at predetermined time points in order to obtain tissue for histologic examination. These time points were initially chosen based upon our past experience with the model and clinical examination of recipient mice using criteria reported to be predictive of rejection in this model (19). Specimens for histologic assessment were fixed in 10% buffered formalin and embedded in paraffin. H&E-stained 3-μm sections were evaluated by a pathologist in a 'blinded' fashion. Rejection was graded according to the following definitions: 0, no rejection; 1, scattered apoptotic crypt cells; 2, focal crypt destruction; and 3, mucosal ulceration with or without transmural necrosis. Full thickness skin grafts (~ 1 cm^2) from the tails of donor mice were transplanted onto the dorsal thorax of recipient mice and secured with a Band-Aid for 7 days. Rejection was defined as the complete loss of viable epidermal graft tissue. Recipient death or graft loss within 7 days of the procedure was considered a technical failure.

Flow cytometric analysis

Peripheral blood was collected, the RBC lysed, and the remaining cells washed with a whole blood lysis kit (R & D Systems, Minneapolis, MN). Peripheral blood leukocytes (PBL) were then stained with the fluorochrome-conjugated Abs anti-CD3, anti-CD11b, anti-GR1, anti-B220, anti-H-$2K^d$, anti-H-$2K^b$, anti-Vβ11, anti-Vβ5.1/5.2, anti-Vβ8.1/8.2 (PharMingen, San Diego, CA), anti-CD4, anti-CD8 (Caltag, Burlingame, CA), or immunoglobulin isotype controls (PharMingen, Caltag). Stained cells were analyzed using CellQuest software on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). Donor chimerism expressed as a percentage that was calculated using the following formula: (H-$2K^{d+}$ cells/total gated cells) × 100.

Allogeneic mixed leukocyte reactions

Purified T cells (R & D systems, Minneapolis, MN) and dendritic cell-enriched transiently adherent splenocytes were used as responders and stimulators, respectively. A total of 10^4 irradiated (2000 rad, ^{137}Cs) stimulator cells were added to 10^5 responder cells in a final volume of 0.20 mL in 96-well round-bottom plates. Proliferation was measured by adding 1 μCi of [^3H] thymidine (Amersham, Arlington Heights, IL)/well after 72 h in culture. The cells were harvested 12–16 h later and counted on a beta-plate counter (LKB Instruments, Gaithersburg, MD). Results are the means of triplicate cultures.

Purification of T cells and T-cell subsets

Splenic $CD3^+$ T cells were purified by negative selection using commercially available murine T-cell isolation columns purchased from R & D Systems. Isolations were performed according to the manufacturer's instructions and resulted in >90% purity as assessed by flow cytometry.

In vivo proliferation assay

Briefly, spleens were harvested and a single-cell suspension was prepared in PBS. Red blood cells were lysed by hypotonic shock. 20×10^6/mL purified T cells (R & D systems) and were then labeled with CFSE (Molecular Probes, Portland, OR) at a final concentration of 10 μM in PBS at room temperature for 10 min Cell labeling was terminated by addition equal volume of FCS for 1 min. Cells were then washed twice in PBS before injection. Each CB6F1 mouse then received 30×10^6 CFSE-labeled cells via the penile vein. After 66 h, splenocytes were harvested from the recipients, and single-cell suspension was prepared after RBC lysis. Cells were stained with PE-conjugated H-$2K^d$ and anti-CD4 (PharMingen) and anti-CD8 (Caltag). Proliferation of CFSE-labeled donor T cells was analyzed by flow cytometry, as above.

Statistical analysis

Rejection grades were compared using the Kruskal–Wallis test for samples from multiple groups and the Mann–Whitney *U*-test for samples from two groups. Continuous variables were compared using the unpaired *t*-test with Welch correction. Calculations were performed using InStat version 2 (GraphPad, San Diego, CA). $p < 0.05$ was considered statistically significant.

Results

Costimulation blockade, busulfan, and donor bone marrow infusion result in multilineage hematopoietic chimerism and promote the long-term acceptance of intestinal allografts. Initial experiments were conducted to confirm that the regimen consisting of costimulation blockade utilizing anti-CD40L mAb, CTLA4-Ig, busulfan, and two infusions of donor bone marrow cells induced multilineage hematopoietic chimerism. Chimerism was initially detected 3–4 weeks after the first infusion of bone marrow cells and was first noted in the B-cell and macrophage compartments (data not shown). Donor granulocytes and T cells were first detected at slightly later time points (data not shown). Having confirmed that this strategy induced chimerism, we sought to determine the effect of this treatment on the survival of intestinal allografts. Following completion of the 6-day treatment regimen, mice were allowed to recover for 8–14 days before undergoing intestinal transplantation. It should be noted that because this is a heterotopic transplant model, the survival of recipient mice is not necessarily dependent upon the survival of the intestinal graft. Thus, in order to determine the status of the graft, recipients were sacrificed at predetermined time points, allowing the grafts to be assessed by both their gross and histologic appearance. As shown in Figure 1(A) the majority of BALB/c intestines transplanted into chimeric C57BL/6 recipients survived long-term. Mild rejection was noted in two of six mice at early time points (50–60 days). Two of seven mice developed severe rejection between 92 and 100 days. Histologic evaluation of the intestinal allografts of the five mice remaining at this later time point revealed normal histology remarkable for the absence of both acute and chronic rejection (Figure 1B). As shown in Figure 2, combined costimulation blockade, busulfan, and bone marrow infusion induced long-term hematopoietic chimerism in the majority of recipients with long-term surviving allografts (14 of 15 mice evaluated between 50 and 100 days). Of note although the majority of mice developed chimerism and experienced long-term survival of the intestinal allografts, the degree of chimerism attained varied significantly between recipients and did not reliably reach the high levels observed following transplantation of nonvascularized (17) or less immunogenic allografts (18). No increase in allograft survival was observed in mice that received costimulation blockade and infusion of bone marrow cells without busulfan relative to untreated recipients. This demonstrates that unlike some other transplant models (20,21) exposure to donor alloantigens under the cover of

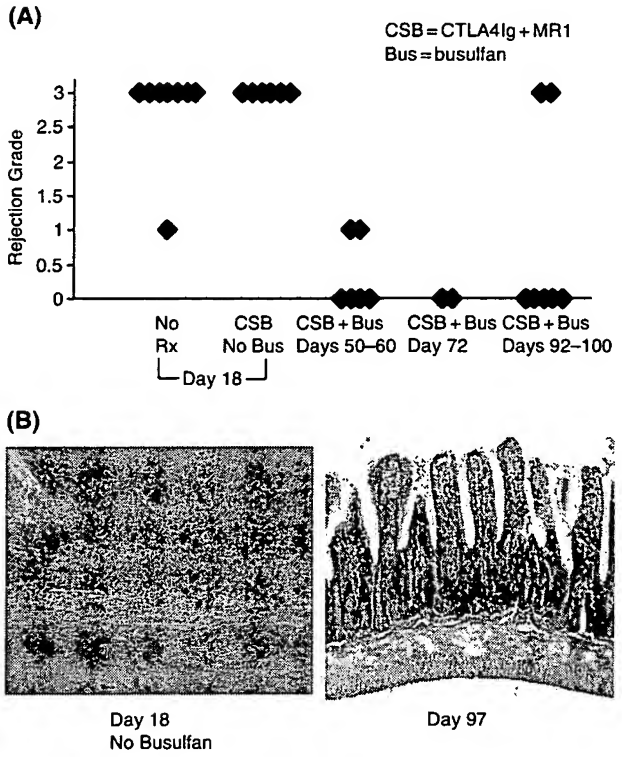

Figure 1: (A) Histologic scores of intestinal allografts from long-term surviving mice following combined costimulation blockade and infusion of donor bone marrow cells. Each symbol (◆) represents the score of an individual recipient. Scores of 0, 1, 2, and 3 denote normal histology, mild, moderate, and severe rejection, respectively. Allografts were assigned a score by a blinded pathologist using the criteria described in the Methods and Materials section. Three mice that survived more than 1 week were found dead of unknown causes precluding histologic graft assessment. (B) Representative H&E-stained sections of intestinal allografts from a recipient treated with combined costimulation blockade and bone marrow without busulfan (day 18) and a recipient treated with combined costimulation blockade, bone marrow, and busulfan (day 97).

costimulation blockade does not significantly inhibit the recipient immune response to intestinal allografts. Lastly, it should be noted that in addition to rejecting the intestinal allograft recipient mice that did not receive busulfan failed to display chimerism (data not shown).

Chimeric recipients of intestinal allografts display donor-specific hyporesponsiveness *in vitro* and delete T cells capable of recognizing MMTV antigens expressed by donor MHC molecules *in vivo*. We next sought to investigate the mechanism responsible for the long-term survival of intestinal allografts in recipients that had been treated using this chimerism-inducing protocol. As shown in Figure 3, T cells from chimeric mice bearing long-term surviving intestinal allografts displayed donor-specific hyporesponsiveness *in vitro*. T cells from the single nonchimeric recipient with a long-term surviving allograft

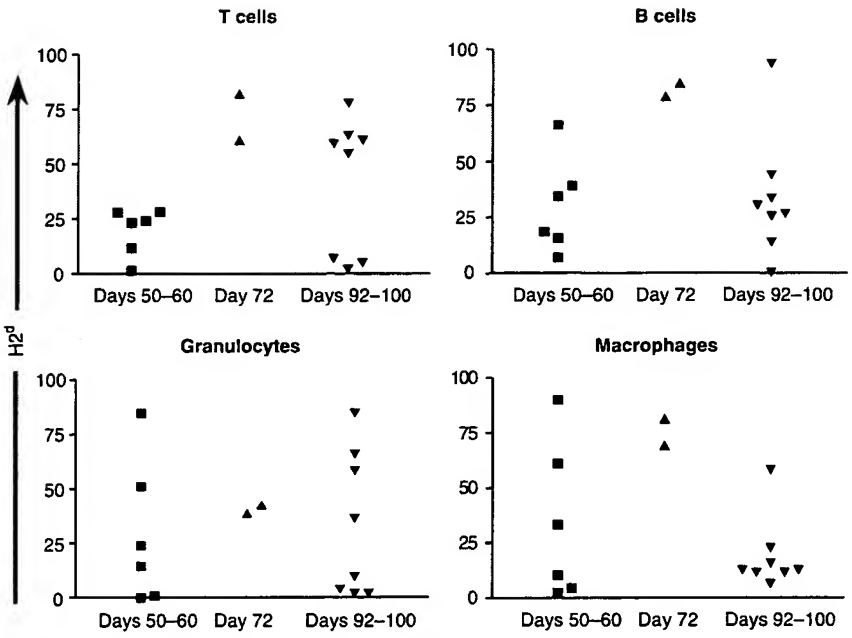

Figure 2: Percent of PBMC that express H-2K^d (donor MHC class I) as detected by flow cytometry. Results shown are for the last sample analyzed before sacrifice of the recipient. Days indicate time since initiation of the chimerism protocol. Each symbol (◆) represents the score of an individual recipient.

displayed a normal response to donor antigen. T cells from mice that were treated with only combined costimulation blockade and infusion of bone marrow cells before intestinal transplantation also proliferated well to donor alloantigen *in vitro*. Several mechanisms could underlie the lack of responsiveness to donor antigens including anergy of donor-reactive cells, the emergence of regulatory cells, and the deletion of donor-reactive T cells. In transplant models where allografts that express I-E are transplanted into recipients that do not express I-E, deletion of CD4+ T cells that express Vβ5 and Vβ11 has been used as a surrogate marker for the presence of T cells capable of responding to donor-strain alloantigens. We found that in C57BL/6 recipients (I-E$^-$) of BALB/c (I-E$^+$) intestinal allografts the frequency of T cells that expressed Vβ5 or Vβ11 following treatment with chimerism-inducing regimen was reduced (1.4±0.7 and 2.8±0.5, n=6) compared with naïve C57BL/6 mice (2.9±0.1 and 4.8±1.6, n=3) (flow cytometric data for an individual mouse from each group is shown in Figure 4). This suggests that deletion contributes significantly to the donor-specific hyporesponsiveness and prolongation of allograft survival that occurs following treatment with this chimerism-inducing regimen. However, even at late time points the frequency of donor-reactive Vα5$^+$ and Vα11$^+$ T cells in chimeric mice bearing BALB/c intestinal grafts had not fallen to the frequencies observed in naïve BALB/c mice (0.2±0.1 and 1.0±0.5, n=3) (Figure 4).

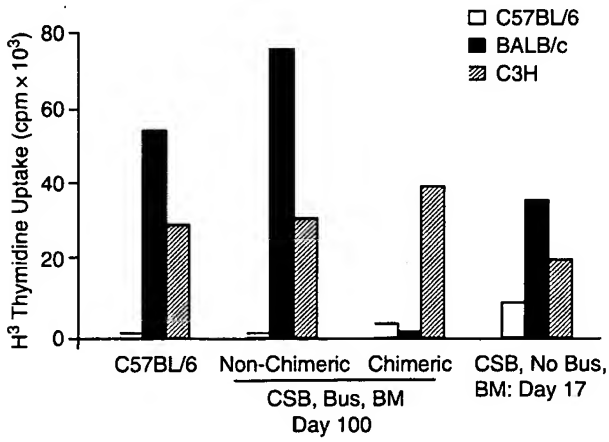

Figure 3: Results of a MLR comparing the proliferation of T cells from (A) a naïve, untransplanted mouse, (B) transplanted mouse that did not receive busulfan and was not chimeric, (C) transplanted mouse that did receive busulfan and was chimeric, and (D) the sole transplanted mouse that did receive busulfan and was not chimeric to self, donor, and third-party antigens. The result shown for the chimeric mouse with a long-term surviving allograft is representative of six recipients.

Recipient T cells that are capable of responding to donor alloantigens persist in chimeric recipients of intestinal allografts. Based on the hyporesponsiveness of recipient T cells to donor alloantigens *in vitro*, the deletion of donor-reactive T cells *in vivo*, and the prolonged survival of intestinal allografts in chimeric mice, we predicted that recipient T cells would also be hyporesponsive to donor antigen *in vivo*. As a first attempt to test this prediction T cells from untransplanted C57BL/6 mice chimeric with respect to BALB/c were studied using an *in vivo* proliferation assay. T cells from chimeric mice, naïve C57BL/6 mice, or naïve CB6F1 mice were labeled with CFSE and injected into CB6F1 mice. As predicted T cells from naïve C57BL/6 mice proliferated well in response to BALB/c alloantigens expressed by the CB6F1 recipient while

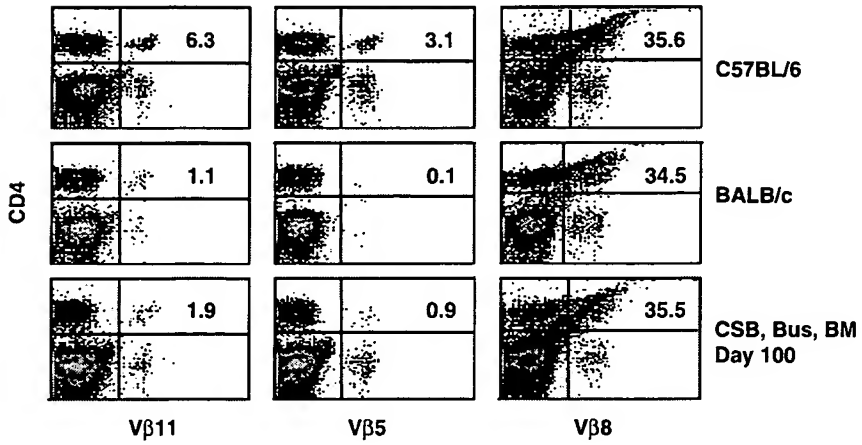

Figure 4: Representative histograms displaying the percentage of T cells expressing Vα5, Vα11, and Vα8.1–2 by T cells isolated from naïve C57BL/6 and BALB/c mice and from a day 100 chimeric C57BL/6 mouse bearing an intestinal allograft. The mean percentage Vα5, Vα11, and Vα8.1–2-positive T cells for each of these groups of mice is stated in the text.

syngeneic CB6F1 T cells did not proliferate when transferred into the F1 recipient (Figure 5). Surprisingly T cells from C57BL/6 mice chimeric with respect to BALB/c also proliferated *in vivo* in response to BALB/c alloantigen, indicating that T cells capable of responding to BALB/c alloantigens persist in chimeric mice. The finding that T cells from chimeric C57BL/6 recipients did not suppress the proliferation of T cells from naïve C57BL/6 recipients suggests that transferable regulatory T cells are not present at the time point examined (Figure 5). These findings raised the possibility that the chimeric mice were not fully tolerant.

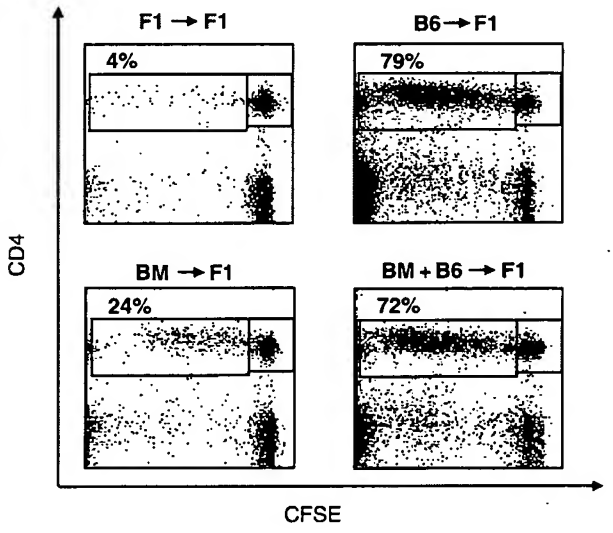

Figure 5: Proliferation of purified, CFSE-labeled T cells from naïve CB6F1/J mice, naïve C57BL/6 mice, chimeric C57BL/6 mice (32 days after the initial dose of combined costimulation blockade and donor bone marrow cells), or a one-to-one mixture of T cells from naïve and chimeric C57BL/6 mice following *in vitro* injection into CB6F1/J hosts. Histograms representative of the results obtained in two experiments (n = 5–6 mice per group total) are shown. In this experiment T cells were pooled from three untransplanted, chimeric C57BL/6 mice (the degree of chimerism detected within the B-cell compartment of these mice was 11%, 17%, and 21%). The percentage of proliferating cells is shown in parenthesis in the left upper quadrant.

In order to determine the consequences of the persistent BALB/c-reactive cells in chimeric mice, mice that had undergone transplantation of BALB/c intestines after receiving the chimerism-inducing regimen including infusion of BALB/c bone marrow cells were challenged with BALB/c (donor) and C3H/HeJ (third party) skin grafts. Grafting was performed at times ranging from 65 to 93 days after the initial infusion of BALB/c bone marrow. Seven of the eight mice were chimeric before skin grafting. As expected, third party skin allografts were promptly rejected. Surprisingly six of the eight skin grafts from BALB/c donors were rejected although their survival was significantly prolonged relative to third party grafts (Figure 6). Rejection of donor-strain skin allografts by chimeric mice bearing intestinal allografts did not result in the loss of chimerism (percent chimerism within the B-cell compartment was 16.8 ± 0.07% before skin grafting vs. 21.8 ± 0.12% after rejection of donor-strain skin grafts). It

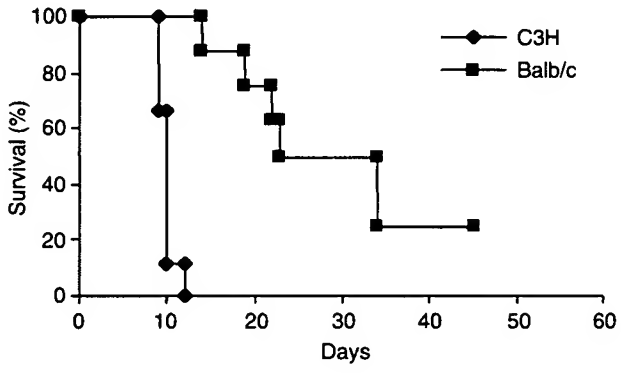

Figure 6: Survival of donor and third-party skin grafts placed on mice bearing intestinal allografts 65–93 days following initial treatment with donor bone marrow cells and combined costimulation blockade.

is also interesting to note that rejection of BALB/c skin grafts by chimeric mice did not invariably result in rejection of intestinal allografts (three mice rejected BALB/c skin grafts but not BALB/c intestinal grafts) although it should be noted that mice were only observed for short periods of time (20–45 days) following rejection of the skin grafts. Together, these finding demonstrate that donor-reactive cells persist in the chimeric mice and that these cells are capable of damaging grafts that express donor alloantigens.

Recipient mice that undergo intestinal transplantation before completion of the chimerism-inducing regimen reject intestinal allografts and fail to develop chimerism. Strategies of this type would be most easily applied clinically if the organ transplant could be performed at the same time that the chimerism-inducing regimen is initiated. We therefore performed a series of intestinal transplants on day 0 of the chimerism-inducing regimen (the day of initial bone marrow cell infusion and treatment with MR1 and CTLA4-Ig). Six of eight recipient mice that underwent intestinal transplantation on day 0 developed severe rejection by days 24–28 (rejection grade = three in all six mice). Furthermore, none of these six mice developed hematopoietic chimerism (data not shown). Of the two mice that did develop chimerism, depletion of donor-reactive Vα5 or Vα11 T cells was minimal 40 days following transplantation (data not shown). These results demonstrate that although prolonged survival and tolerance can be achieved in the mouse skin and heart transplant models when transplantation and chimerism induction begin at the same time (17,18), this is not the case for intestinal allografts in mice.

Discussion

Strategies using the infusion of donor bone marrow cells as a means of establishing mixed allogeneic hematopoietic chimerism and donor-specific tolerance to transplanted organs have been shown to be effective in both rodents and primates (14–17,22–25). Based on these studies trials have been initiated to test the efficacy of this approach in humans. The robust nature of this tolerance prompted us to test the effect of this strategy using a highly immunogeneic murine model of intestinal transplantation. We have previously examined a number of biological agents using this model including monoclonal antibodies specific for CD4, CD8, B7.1, B7.2, CD154, LFA-1, and membrane lymphotoxin and fusion proteins that bind B7, LIGHT, and 4–1BB. Of these agents only the anti-CD4 mAb, which modestly delayed acute rejection, and the anti-CD8 mAb, which inhibited acute but not chronic rejection, were at all effective in wild-type mice (26).

Against this background the finding that a chimerism-inducing strategy comprised of anti-CD154, CTLA4-Ig, busulfan, and donor bone marrow induces long-term survival of fully allogeneic intestinal grafts in the majority of recipients and that these allografts display no histologic features suggestive of chronic rejection represents a significant advance. This observation is consistent with a report that lethally irradiated rats rendered chimeric by the infusion of bone marrow cells failed to develop chronic rejection of intestinal allografts (27). Because in our experiments mice were not followed beyond 100 days, it is possible that chronic rejection could develop later. However, our previous experience with an anti-CD8 mAb demonstrated that chronic rejection of intestinal allografts can develop within 100 days.

While our data demonstrate that combined costimulatory blockade, busulfan, and donor bone marrow infusion is an effective approach for inducing the long-term survival of intestinal allografts, the mechanisms responsible for this effect have not been fully elucidated. Most groups investigating tolerance associated with chimerism-inducing strategies have reported deletion of donor-reactive CD4+ T cells using deletion of MMTV-reactive T cells as a surrogate for alloreactive T cells. It appears that both central and peripheral deletion contribute to this process (28,29). Using the same assay system we have also observed significant deletion of Vβ5+ and Vβ11+ MMTV-reactive CD4+ T cells. While the percentage of CD4+ T cells expressing Vβ5 and Vβ11 was markedly reduced relative to unmanipulated C57BL/6 mice, it was not reduced to the background level measured in unmanipulated BALB/c mice. This suggests that some donor-reactive cells could persist. If this is the case, additional mechanisms would be required to maintain donor-unresponsiveness. These additional mechanisms could include ignorance or anergy of the remaining undeleted cells and/or the development of regulatory T cells.

The need for additional mechanisms capable of controlling the alloresponse of mice treated with this chimerism-inducing regimen is most clearly demonstrated by comparing the outcome of intestinal transplants performed in recipients treated with combined costimulatory blockade, busulfan, and donor bone marrow with those that received only combined costimulatory blockade and donor bone marrow (no busulfan). The majority of recipients in the former group developed chimerism and experienced long-term allograft survival. While this was associated with deletion of donor-reactive cells, depletion was not maximal for at least 40–50 days. The finding that mice treated with only combined costimulation blockade and donor bone marrow all rejected the allograft by day 18 demonstrates that the immunosuppression provided by anti-CD154 and CTLA4-Ig with donor bone marrow is insufficient to prolong the survival of this highly immunogeneic allograft long enough to achieve significant depletion.

If costimulation blockade is not capable of prolonging survival beyond 18 days and deletion is not complete for several more weeks, how is allograft rejection prevented in these mice? Although at this time we can not distinguish definitively between ignorance, anergy, and regulation, the observation that depletion of CD4+ T cells, a potential

regulatory population, prevents the development of chimerism (17) (and data not shown for the intestinal transplant model) suggests that regulation may be important in this model. This hypothesis is consistent with other indirect evidence suggesting a role for regulation in tolerance induced by costimulation blockade and bone marrow infusion (29). Preliminary results from collaborative pilot experiments performed using the same chimerism strategy in a murine cardiac transplant model suggest that at least some recipients do develop regulatory populations of T cells (unpublished data, KAN, Charles Orosz and Alice Gaughan, Ohio State University, Columbus, OH). Although our studies performed using the CFSE *in vivo* proliferation assay failed to demonstrate a population of regulatory T cells, these studies do not exclude the existence of regulatory T cells in these chimeric mice. The *in vivo* CFSE assay only examined the effect of T cells from chimeric mice on the proliferation of alloreactive T cells at one time point. It remains possible that regulatory populations of cells develop in this model at different time points or that they exert their regulatory effect on events other than proliferation such as maturation, effector function, or cell trafficking. Substantially more investigation will be needed to confirm this finding and to determine its relative importance.

Regardless of the mechanism, using different transplant models we and others have reported that regimens that result in macrochimerism produce true tolerance to donor antigens. However, in the current set of experiments we observed prolonged but not indefinite survival of donor-strain skin allografts placed on chimeric mice bearing intestinal allografts. This finding demonstrates that these chimeric mice retain donor-reactive cells that are capable of mediating destruction of allografts expressing donor alloantigens. Therefore these mice are not tolerant by the strictest definition. This observation is consistent with a recent report by Russell et al. in which chimeric mice that had accepted donor-strain skin allografts developed chronic rejection of subsequently placed donor-strain heart allografts (30). The mechanisms underlying this 'split' tolerance remain uncertain. Based on the observation that untransplanted mice rendered chimeric using this protocol accept donor-strain skin grafts placed weeks to months after receiving the chimerism-inducing regimen (17), we do not believe that this finding is a consequence of skin-specific antigens. One potential explanation is that a recently placed nonvascularized skin graft may be more sensitive to a weak ongoing immune response than is an engrafted, vascularized organ. This model would be consistent with the observation that a small number of donor-reactive cells persist in mice following treatment with the chimerism protocol. A second potential explanation is that different types of organs or tissues display a differential susceptibility to injury by a given immunologic mechanism such as has recently been demonstrated for cardiac and skin allografts in mice (31). Although it is unexplained, this observation has important implications for the clinical application of this tolerance strategy.

Although it should be emphasized that the regimen consisting of combined costimulatory blockade, bone marrow infusion, and busulfan results in the best long-term allograft survival of any combination of agents that we have tested in the highly immunogeneic murine intestinal transplant model, our data suggest a potential problem that may affect the clinical implementation of this strategy. We observed that this strategy most often fails to promote chimerism and long-term allograft survival if intestinal transplantation is performed at the time of the initial bone marrow cell infusion. As this scenario reflects how this approach would be applied to humans undergoing transplantation with organs from cadaveric donors, this is a significant limitation. Interestingly, both skin and heart allografts transplanted in mice at the time the chimerism regimen is initiated survive long-term (17,18). We believe that this difference is a consequence of the greater immunogenicity of the intestine compared with the heart or skin. Unlike our initial work, which demonstrated that anti-CD154 and CTLA4-Ig significantly prolonged the survival of skin and heart allografts (32), our subsequent studies as well as data presented in Figure 1(A) show that these agents do not prolong the survival of intestinal allografts (9). Thus we believe that combined costimulatory blockade prolongs the survival of skin and heart allografts long enough for recipients to develop mechanisms that are capable of preventing rejection such as deletion or regulation. In contrast, all intestinal allograft recipients treated with these agents developed severe rejection by day 18; a period of time that is insufficient for deletion to have occurred and appears to also be inadequate for other mechanisms such as regulation to have developed. This line of reasoning suggests that more potent immunosuppressive agents that are compatible with the development of tolerance will need to be identified if bone marrow-based regimens of this type are to be successfully applied to the transplantation of highly immunogeneic organs like the intestine.

In conclusion, while our data demonstrate that this approach can result in the long-term survival of intestinal allografts in some settings, they also demonstrate potential limitations of this regimen such as the high frequency of failure when intestines are transplanted at the initiation of the chimerism regimen and persistence of donor-reactive cells in chimeric recipients. Approaches that overcome these limitations should allow the broader application of this regimen to clinical transplantation. Lastly, our data suggest that mechanisms other than deletion may contribute to the effectiveness of this regimen.

Acknowledgments

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Evidence Appendix 5

U.S. Serial No. 10/057,288

Shirasugi, Nozomu et al., *Journal of Immunology*, 2002, 169:2677-84

Prevention of Chronic Rejection in Murine Cardiac Allografts: A Comparison of Chimerism- and Nonchimerism-Inducing Costimulation Blockade-Based Tolerance Induction Regimens[1]

Nozomu Shirasugi, Andrew B. Adams, Megan M. Durham, Aron E. Lukacher, Huaying Xu, Phyllis Rees, Shannon R. Cowan, Matthew A. Williams, Thomas C. Pearson,[2] and Christian P. Larsen[2]

We have previously described a nonirradiation-based regimen combining costimulation blockade, busulfan, and donor bone marrow cells that promotes stable, high level chimerism, deletion of donor-reactive T cells, and indefinite survival of skin allografts in mice. The purpose of the current study is to determine the efficacy of this tolerance regimen in preventing acute and chronic rejection in a vascularized heart graft model and to compare this regimen with other putative tolerance protocols. Mice receiving costimulation blockade (CTLA4-Ig and anti-CD40 ligand) alone or in combination with donor cells enjoyed markedly prolonged heart graft survival and initially preserved histological structure. However, tolerance was not achieved, as evidenced by the eventual onset of chronic rejection characterized by obliterative vasculopathy and the rejection of secondary skin grafts. In contrast, following treatment with costimulation blockade, busulfan, and bone marrow, heart grafts survived indefinitely without detectable signs of chronic rejection or structural damage, even 100 days after placement of a secondary donor skin graft. We detected multilineage chimerism in peripheral blood, spleen, lymph nodes, and thymus, and peripheral deletion of donor-reactive cells was complete by day 90. These findings indicate that only the CD40/CD28 blockade chimerism induction regimen prevents both acute and chronic rejection of vascularized organ transplants. Further testing of these strategies in a preclinical large animal model is warranted. *The Journal of Immunology,* **2002, 169: 2677–2684.**

Transplantation has become an accepted treatment for patients with end-stage organ failure. However, clinical success of organ transplantation has been achieved through the use of nonspecific immunosuppressive drugs to inhibit immune responses. These drugs generally need to be given for the life of the transplant and have many potential, direct side effects, as well as increased risk of life-threatening complications such as cardiovascular disease, infections, and malignancies. Moreover, while the long-term outcome of transplantation has improved, it remains inadequate (1). The most common cause of late graft loss is chronic rejection, which is characterized by obliterative vasculopathy (2). The most appealing solution to these problems is the induction of transplantation tolerance, defined as lifelong, donor-specific unresponsiveness without the need for chronic immunosuppression. It has been thought that tolerance induction would not only free patients from the morbidity associated with long-term immunosuppression, but also prevent both acute and chronic allograft rejection. However, recent evidence indicates that tolerance induction may not always prevent chronic rejection (3, 4). Thus, it is imperative that careful studies be performed to define the effects of specific tolerance induction regimens on the development of chronic rejection.

In recent years, several clinically relevant immunomodulatory or tolerance induction regimens have been reported that incorporate the use of Abs and/or fusion proteins that target the CD28/B7 and CD40/CD154 pathways with or without the concomitant administration of donor cells (splenocytes or bone marrow cells (BMCs)[3]) (5–9). Although the immunological mechanisms underlying the effectiveness of these regimens are incompletely understood, recent advances have suggested that the development of tolerance involves the participation of $CD4^+$ regulatory T cells that are activated or exert their function in a CTLA-4-dependent manner. Furthermore, evidence suggests that the continued presence of a vascularized allograft can itself play a critical role in tolerance maintenance (10). Although several of these studies have indicated costimulation blockade (CB)-based regimens can inhibit chronic rejection, the duration of follow-up and the tests of the stability of tolerance in these studies provide insufficient data upon which to draw firm conclusions about long-term outcomes (5–7).

The induction of mixed hemopoietic chimerism has been a promising strategy for the induction of robust immunological tolerance for many years (11, 12). Although in the past most chimerism induction regimens required the use of gamma irradiation and/or depletion of the peripheral immune system (13–17), more recently, protocols using either the administration of mega doses of

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[3] Abbreviations used in this paper: BMC, bone marrow cell; Bu, busulfan; CB, costimulation blockade; CD40L, CD40 ligand; DST, donor splenocyte; MST, median survival time.

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donor bone marrow or the use of minimally myelosuppressive recipient conditioning combined with various forms of CB have increased the likelihood that clinically acceptable tolerance induction regimens based on these principles can be devised (18, 19). The strategy that we have used incorporates the CTLA4-Ig fusion protein to target the CD80/CD86-CD28 pathway and MR1, an anti-CD154 mAb to target the CD40-CD40 ligand (CD40L) interactions, and the administration of donor BMCs after conditioning the recipient with a minimally myelosuppressive dose of the chemotherapeutic conditioning agent, busulfan (Bu). This regimen induces high level, stable mixed hemopoietic chimerism and specific deletional tolerance to fully MHC-mismatched allogeneic skin grafts (20).

Due to the limitations of the skin allograft model, our earlier studies were unable to address the important issue of degree of protection conferred from acute cellular infiltration and chronic rejection. Because of the increasing evidence that tolerance may not equate to freedom from chronic rejection, we used the murine cardiac allograft model to compare the ability of this and other promising tolerance induction regimens to protect vascularized allografts from immunologic injury during tolerance induction and to prevent chronic rejection. The need for such studies is underscored by the report by Russell et al. (4) that was published during the preparation of this work that demonstrated that even the induction of robust tolerance using chimerism induction strategies may not always be sufficient to prevent chronic rejection.

In this study, we compare the effects of several clinically relevant CB-based tolerance induction regimens to promote cardiac allograft survival, to induce donor-specific tolerance, and to prevent acute and chronic allograft rejection. We find that treatment regimens consisting of CB alone (CTLA4-Ig and anti-CD40L), CB and donor BMCs, and CB and donor splenocytes (DST) promote long-term allograft survival, but do not confer robust tolerance nor prevent chronic rejection in the face of a rechallenge with a donor skin graft. In contrast, a regimen consisting of CTLA4-Ig, anti-CD40L, donor BMCs, and a minimally myelosuppressive dose of Bu produced stable donor-specific tolerance, and prevented both early and late cellular infiltration and chronic allograft vasculopathy, despite the rigorous rechallenge of a donor skin graft.

Materials and Methods

Mice

Adult male 6- to 8-wk-old C57BL/6 (B6) (H-2^b), BALB/c (H-2^d), and C3H/HeJ (H-2^k) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in specific pathogen-free conditions and in accordance with institutional guidelines.

Bone marrow preparation and treatment regimens

Bone marrow was flushed from tibiae, femurs, and humeri. Red cell lysis was performed using a Trizma base ammonium chloride solution. The donor BMCs were resuspended at 2×10^7 cells/250 μl sterile saline and injected i.v. on the day of cardiac transplantation (day 0). Hamster anti-mouse CD40L (MR1; Bioexpress, Lebanon, NH) and CTLA4-Ig (Bristol-Myers Squibb, Princeton, NJ) were administered on days 0, 2, 4, 6, 14, 28, 60, and 90 (500 μg/dose i.p., respectively). The recipients received Bu (20 mg/kg, i.p.) on day 5 and second dose of donor BMCs (i.v.) on day 6 (20). Mice in other groups received CB consisting of anti-mouse CD40L and CTLA4-Ig alone, CB with two doses of donor BMCs (without Bu), or CB with two doses of DST (2×10^7 cells on days 0 and 6).

Heart grafting

Fully vascularized heterotopic hearts from BALB/c donors were transplanted into the abdomen of B6 recipients using microsurgical technique on day 0, as previously described (21). Graft survival was followed by palpation at least three times per week. Rejection was defined by complete cessation of palpable contraction confirmed by direct visualization. Histological examination was also performed to confirm the condition of the grafts.

Skin grafting

Full thickness skin grafts (~1 cm^2) were transplanted on the dorsal thorax of recipient mice and secured with a Band-Aid for 7 days. Rejection was defined as the complete loss of viable epidermal graft tissue.

Flow cytometric analysis

We performed multicolor flow cyotmetry. Donor and recipient cells were distinguished by staining with anti-H-$2K^d$ and anti-H-$2K^b$, respectively. To analyze the degree and distribution of hemopoietic chimerism in multiple compartments and in multiple organs in the recipient, lineage-specific markers such as anti-B220 (for B cells), anti-CD4, anti-Gr1 (for granulocytes), anti-CD11b (for monocytes/macrophages), and CD11c (for dendritic cells) (22, 23) were used. Peripheral blood was analyzed by staining with fluorochrome-conjugated Abs (anti-CD11b, anti-GR1, anti-B220, anti-CD8, anti-H-$2K^d$, anti-H-$2K^b$, anti-Vβ11, anti-Vβ5.1/5.2, anti-Vβ8.1/8.2 (BD PharMingen, San Diego, CA), anti-CD4 (Caltag Laboratories, Burlingame, CA), or Ig isotype controls (BD PharMingen)), followed by RBC lysis and washing with a whole blood lysis kit (R&D Systems, Minneapolis, MN). Single cell suspensions of spleen, abdominal lymph nodes, thymus, or bone marrow were also analyzed by staining with fluorochrome-conjugated Abs (anti-B220, anti-CD4, anti-CD8, anti-H-$2K^d$, anti-H-$2K^b$, anti-Vβ11, anti-Vβ5.1/5.2, anti-Vβ8.1/8.2, or Ig isotype controls) after RBC lysis with a Trizma base ammonium chloride solution.

Dendritic cell-enriched populations were prepared as transiently adherent cells from spleen, lymph nodes, thymus, or bone marrow, as previously described (24), and stained with fluorochrome-conjugated Abs (anti-CD11c, anti-H-$2K^d$, anti-I-A^d (BD PharMingen), or Ig isotype controls). Stained cells were analyzed using CellQuest software on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA).

Graft histology

Fresh tissues were fixed in 4% paraformaldehyde until processed and embedded in paraffin (Fisher Scientific, Pittsburgh, PA). Five-micron-thick tissue sections were cut on a microtome and stained with H&E or Masson's trichrome, according to standard procedures. To evaluate the degree of chronic rejection, the number of vessels, including coronary arteries and i.m. arterioles affected with obliterative vasculopathy, and the total number of vessels in each section of the histological specimen were counted. Histological specimens were reviewed by a single histologist (H. Xu) blinded to the treatment modality.

Statistical analysis

Graft survival between groups was analyzed by Mann-Whitney *U* test using Stat View 5.0 software (Abacus Concepts, Berkeley, CA). Statistical significance of other data was analyzed by unpaired Student's *t* test using Stat View 5.0.

Results

We have recently reported that a regimen consisting of CB, donor BMCs, and a minimally myelosuppressive, nonablative dose of Bu induces high level mixed hemopoietic chimerism and transplantation tolerance in a rigorous mouse skin graft model (20). The purpose of this study was to compare the effects of this regimen with three putative tolerance induction strategies, including CB alone (anti-CD40L and CTLA4-Ig), CB with BMCs, and CB with DST, on 1) cardiac allograft survival, as assessed by the persistence of a palpable heartbeat; 2) the ability to induce robust tolerance, as assessed by the ability to accept a secondary donor skin graft; 3) the ability to protect allograft from acute and chronic rejection, as assessed by histopathological examination; and 4) the kinetics of the development of hemopoietic chimerism and deletion of donor-reactive T cells, as assessed by flow cytometry.

Effects on cardiac allograft survival: all CB-based regimens greatly prolong survival of mouse primary cardiac allografts

As seen in Fig. 1, untreated B6 recipients or recipients treated with isotype control Abs rejected BALB/c heart grafts rapidly (median survival time (MST) = 8 days, n = 10 and 5, respectively).

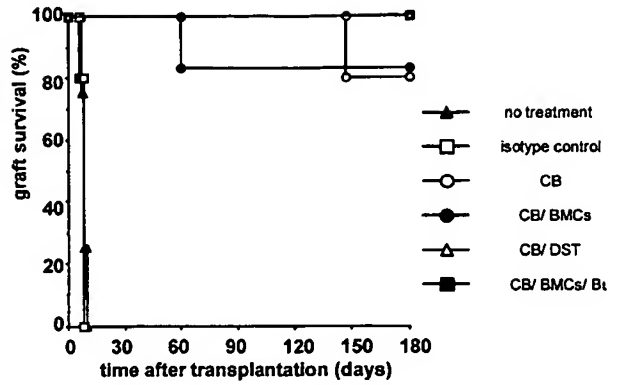

FIGURE 1. CB with CTLA4-Ig and anti-CD40L induces long-term acceptance of cardiac allografts. B6 (H-2^b) recipients of BALB/c (H-2^d) cardiac grafts received CB, BMCs, Bu (■, $n = 5$); CB, DST (△, $n = 4$); CB, BMCs (●, $n = 5$); CB (○, $n = 6$); isotype control (□, $n = 5$); or no treatment (▲, $n = 10$). All animals (B6) received fully allogeneic cardiac grafts (BALB/c) on day 0. Control groups (no treatment, or isotype control) promptly rejected the BALB/c cardiac grafts (MST = 8 days, respectively). Recipients treated with CB-based regimens (i.e., recipients with CB alone; CB and BMCs; CB and DST; or CB, BMCs, and Bu) showed prolonged survival of primary cardiac allografts with MST >180 days, while one recipient each from group with CB alone or with CB and BMCs rejected the grafts on days 147 and 60, respectively. Additional groups performed with these regimens yielded similar survival results, but were harvested for histologic assessment on day 14, 28, 60, or 90.

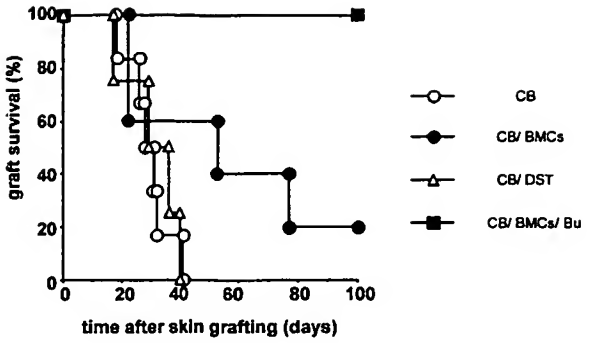

FIGURE 2. Only administration of CB, BMCs, and Bu promotes the development of transplantation tolerance in allogeneic recipients. Two hundred days after primary cardiac grafting, recipients with accepted cardiac grafts received secondary donor-specific (BALB/c, H-2^d) and third party (C3H, H-2^k) skin grafts. Recipients with CB alone (○, $n = 5$), CB and BMCs (●, $n = 4$), or CB and DST (△, $n = 4$) eventually rejected secondary BALB/c skin grafts with MSTs of 30, 53, and 33 days, respectively. In contrast, BALB/c skin grafts placed on animals treated with CB, BMCs, and Bu (■, $n = 5$) survived indefinitely (>100 days). All recipients rejected third party (C3H) skin grafts within 10–15 days (data not shown).

BALB/c hearts were also acutely rejected by B6 recipients receiving only donor BMCs (MST = 9 days, $n = 6$), by those receiving donor BMCs and Bu (MST = 10 days, $n = 5$), and by those treated with Bu only (MST = 10 days, $n = 4$) (data not shown). In contrast, groups treated with CB (i.e., recipients with CB alone ($n = 6$); with CB and BMCs (no Bu) ($n = 5$); with CB and DST ($n = 4$); or with CB, BMCs, and Bu ($n = 5$)) showed long-term survival of BALB/c heart grafts (MST >180 days in all these groups) (Fig. 1). There was no statistically significant difference in BALB/c heart survival among these groups.

Effects on tolerance induction: only recipients of CB, Bu, and donor BMCs develop stable transplantation tolerance

Next, to determine whether recipients were rendered tolerant, recipients in the various treatment groups with long-term surviving BALB/c heart grafts were rechallenged with secondary skin grafts from BALB/c (donor-specific) and C3H (third party) donors 200 days after primary heart transplantation. Recipients that had received CB, BMCs, and Bu uniformly accepted second BALB/c skin grafts (MST >100 days). In contrast, recipients treated with CB and BMCs, CB and DST, or CB alone showed evidence of donor-specific hyporesponsiveness, but eventually rejected second BALB/c skin grafts (MST = 53, 33, and 30 days; $n = 4$, 4, and 5, respectively) (Fig. 2). Recipients from all groups promptly rejected C3H (third party) skin grafts (MSTs = 14 days) (data not shown).

Effects on acute and chronic rejection—histopathological analysis: CB-based regimens prevent early tissue damage, but only the regimen of CB, Bu, and donor BMCs prevents chronic rejection of heart allografts

The most commonly used endpoint for the assessment of murine heterotopic cardiac allograft survival is the cessation of a palpable heartbeat. To gain better insight into the degree to which the various regimens protected the allografts from immunologic injury (acute and chronic rejection), we performed detailed histological examinations of these BALB/c heart grafts using H&E and Masson's trichrome staining at 14, 28, 60, 90, and 300 (i.e., 100 days after secondary skin grafts) days after heart transplantation or at the time of allograft failure. Allografts harvested at time points up to 90 days posttransplant (while treatment with CB is ongoing) were free of myocardial injury in all groups receiving CB (CB alone (Fig. 3*D*); CB and BMCs (Fig. 3*E*); CB and DST (Fig. 3*F*); or CB, BMCs, and Bu (Fig. 3*C*)). However, allografts treated with CB alone or CB and DST had rare sparse interstitial infiltrates, whereas allografts treated with CB, BMCs, and Bu were indistinguishable from syngeneic grafts (Fig. 3*B*). Grafts harvested on day 14, 28, or 60 in these recipients (either treated with CB alone; CB and BMCs; CB and DST; or CB, BMCs, and Bu) had similar histology to those harvested on day 90 (data not shown). These data suggest that prolonged treatment with anti-CD40L and CTLA4-Ig can protect vascularized murine cardiac allografts during the induction phase of these regimens.

The long-term outcomes (300 days) 100 days after rechallenge with a secondary donor skin graft were strikingly different. Allografts in recipients with CB only (Fig. 4*D*), with CB and BMCs (Fig. 4*E*), or with CB and DST (Fig. 4*F*) showed typical changes characteristic of severe chronic rejection, including severe interstitial fibrosis, diffuse infiltration of mononuclear cells, and obliterative vasculopathy with diffuse intimal thickening in coronary arteries in the grafts. The number of coronary arteries and i.m. arterioles affected with obliterative vasculopathy in these three groups ranged from 64 to 87% of vessels observed (Table I). This occurred despite the continued survival of the grafts as assessed by palpation. Examples of normal cardiac histology (Fig. 4*A*) and BALB/c cardiac allograft undergoing acute rejection in an untreated B6 recipient 10 days after transplantation (Fig. 4*B*) are shown for comparison.

In contrast, the grafts in mice receiving the chimerism induction regimen of CB, BMCs, and Bu did not show any evidences of either acute or chronic rejection (Fig. 4*C*). In the recipients of CB, BMCs, and Bu, cardiac allografts were morphological normal and remained free of interstitial or vascular pathology, similar to that of naive BALB/c heart (Fig. 4*A*). No vessels with obliterative vasculopathy were observed in specimens (10 coronary cross sections/specimen) from recipients treated with CB, BMCs, and Bu (Table

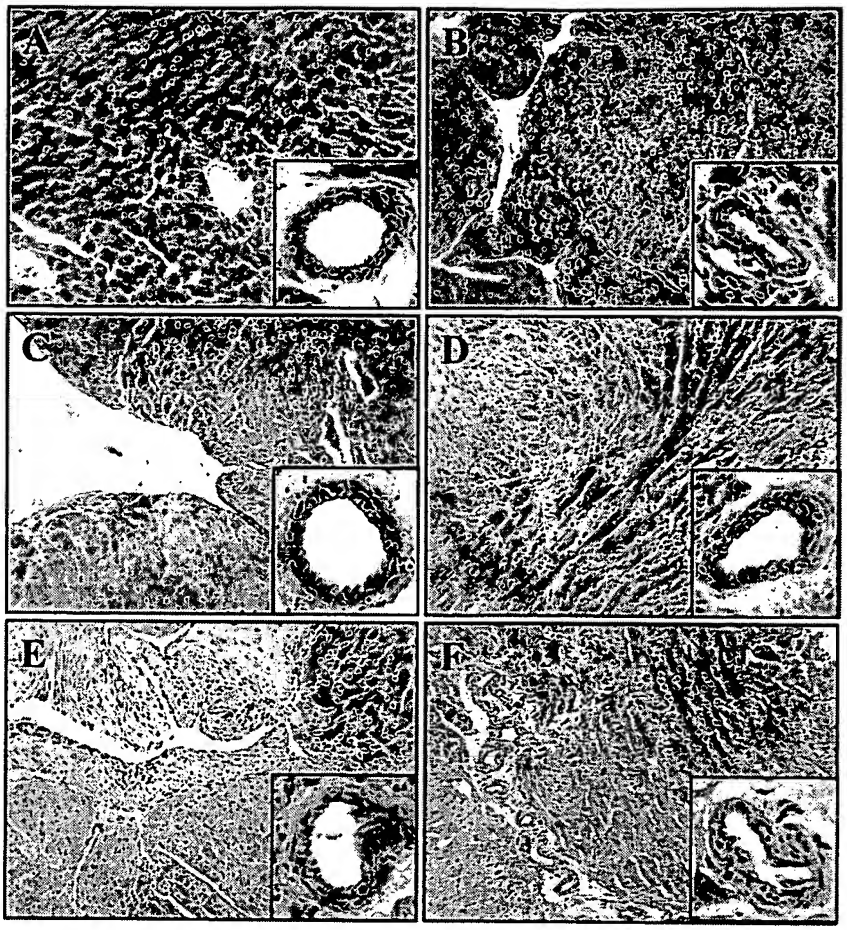

FIGURE 3. Morphology of cardiac grafts at day 90. Cardiac grafts were harvested from B6 recipients on day 90 after transplantation. Tissue sections were stained with H&E. *A*, BALB/c naive heart for comparison. *B*, Syngeneic graft in a B6 recipient on day 90 showing normal heart architecture with little leukocyte infiltration. There is no difference in histology between B6 and BALB/c syngeneic grafts (data not shown). *C*, BALB/c allograft in B6 recipient treated with CB, BMCs, and Bu. *D*, BALB/c allograft in B6 recipient treated with CB alone. *E*, BALB/c allograft in B6 recipient treated with CB and BMCs (no Bu). *F*, BALB/c allograft in B6 recipient treated with CB and DST on day 90. Histology of these grafts (*C–F*) shows that normal heart architecture is preserved. There is no evidence of transplant vasculopathy in coronary vessels in these grafts. However, allografts with CB, BMCs, and Bu have a few infiltrating leukocytes, much like the syngeneic grafts, while allografts with CB alone (*D*) or with CB and DST (*F*) demonstrate somewhat more extensive infiltration in the interstitial space. Similar histological results were obtained from three allografts from each experimental group (H&E; magnification ×200; insets ×400).

I). Very similar results were observed in all groups when recipients were treated with CB on days 0, 2, 4, 6, 14, and 28. We have not tested shorter treatment regimens.

Effects on recipient immune system: analysis of the distribution and kinetics of hemopoietic chimerism

Next, we compared the degree and distribution of hemopoietic chimerism in the recipients of each regimen. As expected, only treatment with CB, BMCs, and a nonmyeloablative dose of Bu could induce multilineage hemopoietic chimerism in peripheral blood of recipients after day 14 (Fig. 5, *A–C*). Similar levels of donor chimerism were observed among $B220^+$, $CD4^+$ cells, and $CD11c^+$ dendritic cells in spleen (Fig. 5*D*), abdominal lymph node (Fig. 5*E*), bone marrow (Fig. 5*F*), and thymus (Fig. 5*G*). Recipients with no treatment, donor BMCs alone, donor BMCs and Bu, or CB alone had no detectable hemopoietic chimerism in any organs (data not shown). Recipients treated with CB and donor BMCs (no Bu), or with CB and two doses of DST showed minimal donor chimerism on day 14 and virtually undetectable levels of donor cells thereafter, suggesting that those donor cells were not engrafted populations, but only passenger leukocytes (Fig. 5, *A* and *B*).

Effect on recipient immune system: sustained deletion of Vβ11 and Vβ5 T cells only occurred with hemopoietic chimerism

As a measure of the ability of these regimens to reshape the recipients' peripheral T cell repertoire, we tracked the fate of the mouse mammary tumor virus superantigen-reactive T cells as a surrogate marker for antidonor reactive T cells (16, 18, 20). Donor BALB/c mice express mouse mammary tumor virus-8 and 9 in association with MHC class II I-E molecules and delete Vβ11- and Vβ5-bearing $CD4^+$ T cells, whereas recipient B6 mice do not express I-E and use Vβ11 on ~4–5% of $CD4^+$ T cells and Vβ5.1/2 on ~2–3% of $CD4^+$ T cells (25, 26). As anticipated, recipients treated with donor BMCs alone, with donor BMCs and Bu, or with Bu alone failed to delete donor-reactive $V\beta11^+$ or $V\beta5^+CD4^+$ T cells (data not shown). Recipients treated with CB and BMCs (no Bu) showed a transient decrease in the percentage of $CD4^+V\beta11^+$ and $CD4^+V\beta5^+$ T cells on day 28, but these populations recovered by day 60. This effect was not observed in recipients treated with CB alone (not shown) or CB and DST (Fig. 6, *A* and *B*). In contrast, the recipients with CB, BMCs, and Bu developed profound deletion of $CD4^+V\beta11^+$ and $CD4^+V\beta5^+$ T cells in peripheral blood (Fig. 6*C*), spleen (Fig. 6*D*), and abdominal lymph nodes (Fig. 6*E*). The deletion process with this regimen took place over a surprisingly long period of time. Virtually no peripheral deletion of $CD4^+V\beta11^+$ T cells was evident in blood, spleen, or lymph node at day 14. Only ~50% depletion was achieved by day 28, and near complete deletion occurred between days 60 and 90. Similar, but slightly more rapid and complete deletion (day 28) of $CD4^+V\beta5^+$ T cells was also observed. Central deletion of $CD4^+$ ($CD8^-$) $V\beta11^+$ and $CD4^+$ ($CD8^-$) $V\beta5^+$ thymocytes was only observed in the recipients treated with CB, BMCs, and Bu, but this deletion was not apparent until day 60 (Fig. 6*F*). The percentage of Vβ8-bearing $CD4^+$ T cells, which are expressed on ~15–20% of BALB/c and B6 $CD4^+$ T cells, was similar in all groups, indicating that the T cell deletion was donor specific in nature (data not shown).

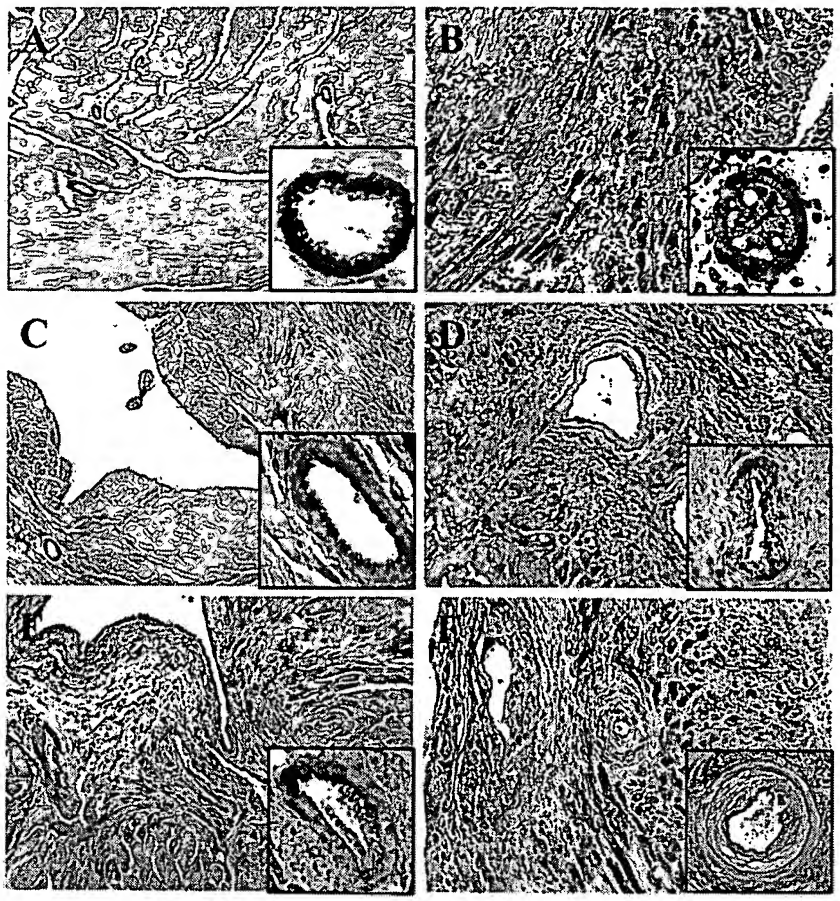

FIGURE 4. Morphology of cardiac allografts on day 300 after transplantation. Cardiac allografts that were not rejected 100 days after secondary skin grafting (i.e., the allografts with palpable beating 300 days after primary cardiac transplantation) were harvested from B6 recipients treated with either CB alone; CB and BMCs; CB and DST; or CB, BMCs, and Bu. Tissue sections were stained with Masson's trichrome. *A*, BALB/c naive heart for comparison. *B*, BALB/c allografts in B6 recipients on day 10 without any treatment for comparison. In this allograft, findings are consistent with acute rejection, including diffuse lymphocytic infiltration with myocyte damage. Obliterative vasculopathy was not seen. *C*, BALB/c allograft in B6 recipient treated with CB, BM, and Bu. *D*, BALB/c allograft in B6 recipient treated with CB alone. *E*, BALB/c allograft in B6 recipient treated with CB and BMCs (no Bu). *F*, BALB/c allograft in B6 recipient treated with CB and DST. Histology of allografts in recipients with CB alone, CB and BMCs, or CB and DST (*D–F*) showed extensive fibrosis in the grafts (collagen is highlighted as blue in Masson's trichrome stain), as well as infiltration of mononuclear cells in the interstitial space, especially adjacent to the coronary vessels. Coronary vessels (in insets) showed intimal hyperplasia and resulting narrowing of the lumen, characteristic of the obliterative vasculopathy of chronic rejection. There is no difference in spectrum of vascular pathology in these three groups (*D–F*). In contrast, histology of the allografts in the recipients treated with CB, BMCs, and Bu showed preservation of normal myocyte architecture and normal coronary vessel structure. These grafts were essentially free from any infiltrate and obliterative vasculopathy (*C*). Similar histological results were obtained from three allografts from each experimental group (Masson's trichrome; magnification ×200; insets ×400).

Discussion

We have recently demonstrated that a regimen consisting of CB, a nonmyeloablative dose of Bu, and donor BMCs induces high level, stable hemopoietic chimerism and robust transplantation tolerance in the mouse skin graft model (20). The skin graft model was used in these initial studies because it is an immunologically stringent test for the efficacy of this approach. However, unlike organ allografts, the skin model is not primarily vascularized and does not offer a well-characterized method to assess the degree of immunologic injury sustained by the allograft. Given the numerous reports that tolerance protocols can be associated with active immunologic infiltrates (9, 27), and or transplant vasculopathy, we felt that it was important to determine the ability of this combined CD40/CD28 CB chimerism induction protocol to protect allografts from acute and chronic rejection using the primarily vascularized mouse cardiac allograft model. In addition, we have performed a long-term comparison with other regimens that we and others have described that can also promote long-term survival of primary cardiac allografts.

Table I. *Analysis of the heart grafts 300 days posttransplant*[a]

Treatment	No. of Grafts with Vasculopathy	No. of Arteries and Arterioles with Vasculopathy[b]
CB	5/5	9.27 ± 1.39/14.5 ± 1.60
CB and BMCs	4/4	10.5 ± 2.39/13.1 ± 1.92
CB and DST	4/4	10.8 ± 1.61/12.4 ± 2.02
CB, BMCs, and Bu	0/5	0/14.3 ± 1.46

[a] The number of vessels, including coronary arteries and intramuscular arterioles, with vasculopathy in the heart grafts 300 days after transplantation, was analyzed by a single reviewer blinded to the treatment modality.

[b] The number of the vessels with vasculopathy/the total number of coronary arteries and i.m. arterioles in the graft. The data represent the mean and the SD for each group.

Not surprisingly, we found no statistically significant difference in primary cardiac graft survival (as assessed by graft palpation) or cardiac allograft histology (before secondary rechallenge of alloantigen) between the regimen inducing mixed chimerism and other regimens including CB alone, CB and BMCs, or CB and

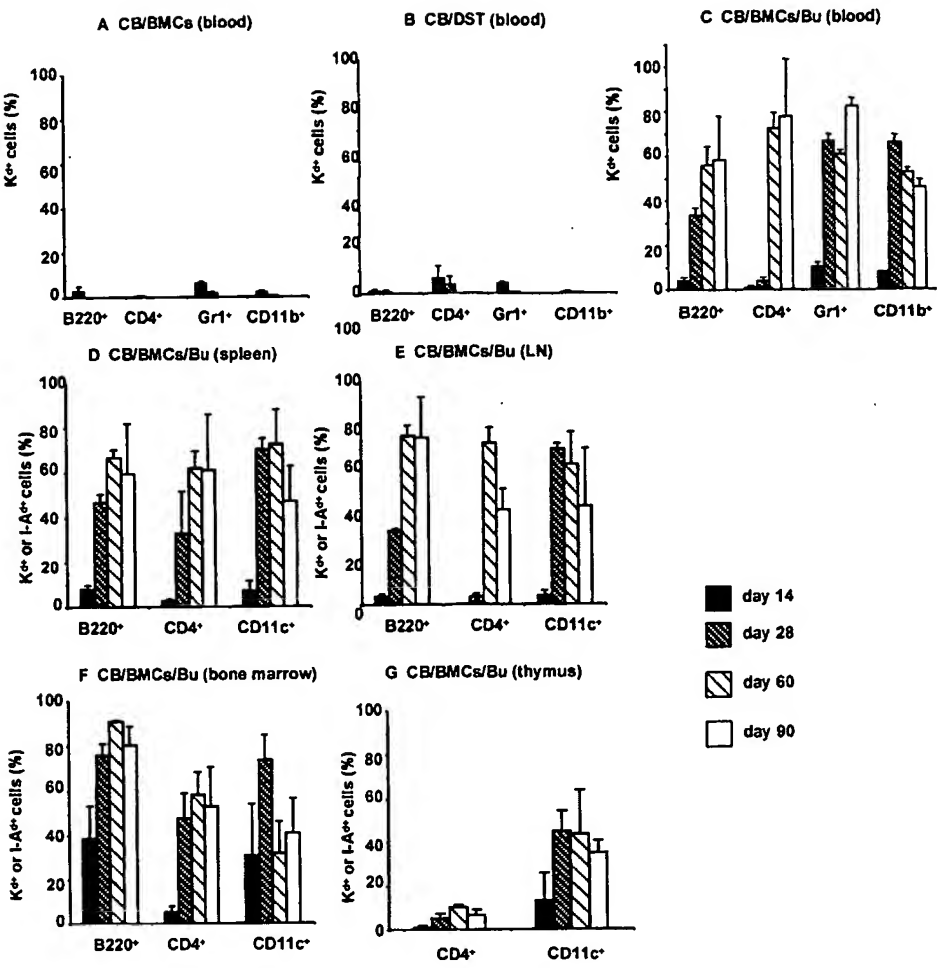

FIGURE 5. Degree and distribution of chimerism in peripheral blood, the spleen, abdominal lymph nodes, bone marrow, and the thymus. Donor representation among various hemopoietic lineages (B cell, $CD4^+$ cell, granulocyte, monocyte/macrophage, and dendritic cell compartments) was determined on days 14, 28, 60, and 90 after primary heart grafting in peripheral blood (*A–C*), the spleen (*D*), abdominal lymph nodes (*E*), the bone marrow (*F*), and the thymus (*G*) by flow cytometry. Only treatment with CB, BMCs, and Bu promotes multilineage chimerism in peripheral blood (*C*), spleen (*D*), lymph nodes (*E*), bone marrow (*F*), and thymus (G), while recipients treated with CB and BMCs (*A*), or CB and DST (*B*) showed transient and less than 5% donor cells only detected within 28 days of transplantation. All B6 recipients received fully allogeneic cardiac grafts (BALB/c, H-2^d) on day 0. Data points represent the mean and the SD for each group (n = 3) ($B220^+$, B cell compartment; $CD4^+$, $CD4^+$ cell compartment; $Gr1^+$, granulocyte compartment; $CD11b^+$, monocyte/macrophage compartment; $CD11c^+$, dendritic cell compartment).

DST (Figs. 1 and 3). For example, on day 90, grafts from all of the groups that received CB continued to beat and showed only rare infiltrating cells equivalent to that observed in syngeneic grafts in the control group. Consistent with our earlier work, and that of others, these data suggest that the combination of CTLA4-Ig and anti-CD40L can protect cardiac allografts from acute rejection for relatively prolonged periods. These data are also important in that they demonstrate that enhanced graft survival that results from this regimen of prolonged CB does not require the presence of a lymphocytic infiltrate of a significant number of regulatory cells. Although this does not exclude a role for a small number of regulatory cells in the graft or a role in the lymphoid tissues, this knowledge is of practical significance for setting expectations for protocol allograft biopsies that might be obtained in the conduct of clinical trials (i.e., an infiltrate cannot be presumed to be an obligatory feature of the enhanced survival of immunotherapies directed at T cell costimulation).

In contrast to the effects on primary graft survival, only the chimerism induction regimen (CB, BMCs, and Bu) prevented cardiac allograft rejection after rechallenge with a secondary donor skin graft. It is reasonable to question the importance of the finding that the protocols differ in their ability to protect from rejection that is only evident after a rechallenge with a donor-specific skin graft, as this scenario would not occur in the clinical setting. However, studies conducted using mice housed in highly protected specific pathogen-free conditions also may not accurately reflect the stability of tolerance under normal external environmental circumstances. For example, it is known that T cells specific for pathogens overlap with the allospecific repertoire (28, 29). In the specific pathogen-free conditions used for most murine tolerance studies, activation of cross-reactive T cells might not occur often, if at all, whereas in a clinical setting it is possible, if not likely, that immune responses to environmental pathogens might involve alloreactive cells that could precipitate rejection of grafts maintained by the more tenuous mechanisms that sustain allograft survival in the nonchimerism-based regimens tested in this study. It is noteworthy that one recipient in both the groups receiving CB only and the CB and BMCs group rejected BALB/c grafts with histology typical of severe chronic rejection before placement of the secondary skin graft. Additionally, the sustained presence of the vascularized cardiac graft was not sufficient for the induction of robust tolerance that has been observed with other tolerance regimens (10). In contrast, in the chimeric mice receiving CB, BMCs, and Bu, the inability of donor rechallenge to precipitate rejection suggests that viral infections are unlikely to perturb tolerance established by this regimen. Indeed, although graft histology was not assessed in our skin graft studies, acute lymphocytic choriomeningitis virus infection failed to promote overt rejection of skin grafts after the establishment of hemopoietic chimerism (30).

The findings of this study are in apparent conflict with several reports that have suggested that CB or CB with donor cells can prevent chronic rejection (5, 6, 31–33). For example, we have previously reported that simultaneous blockade of CD28 and CD40 pathways inhibits the chronic transplant vasculopathy in BALB/c heart grafts in C3H recipients (5), and other groups have demonstrated that blockade of these pathways inhibits transplant vasculopathy in C57BL/10 aortic grafts in C3H recipients (31), rat kidney (32), and heart allograft (33). In addition to role of the

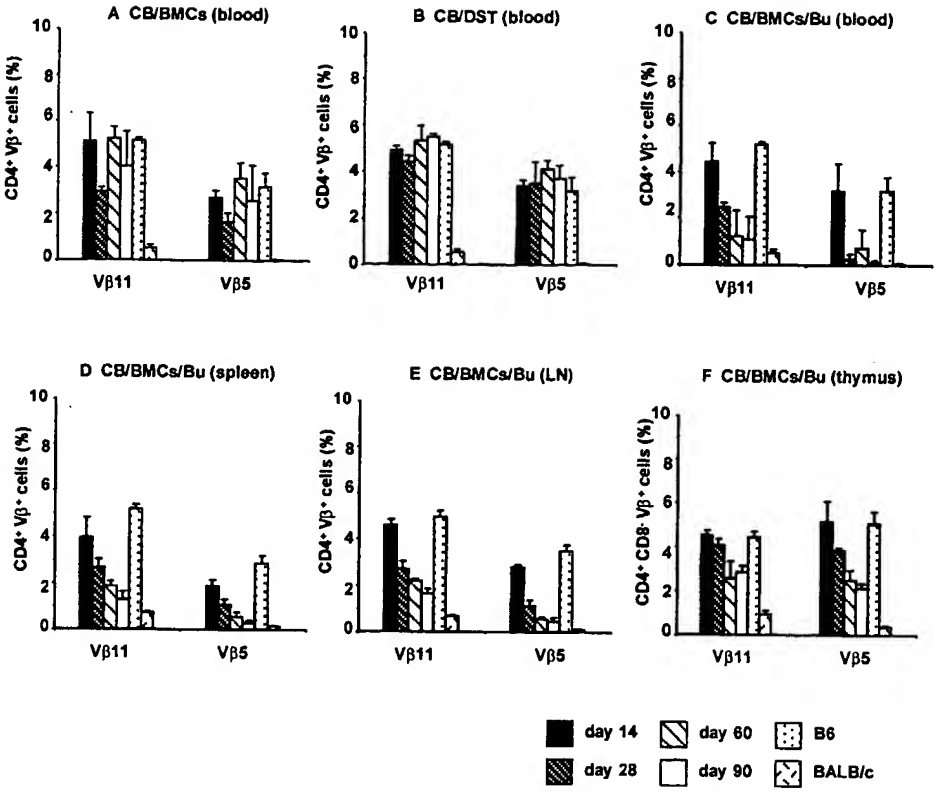

FIGURE 6. Deletion of donor-reacting T cells in peripheral blood, the spleen, abdominal lymph nodes, and the thymus. Deletion of the $CD4^+V\beta11^+$, $V\beta5.1/2^+$, and $V\beta8.1/2^+$ T cells was determined on days 14, 28, 60, and 90 after primary heart grafting in peripheral blood (*A–C*), the spleen (*D*), abdominal lymph nodes (*E*), and the thymus (*F*) by flow cytometry. Only recipients treated with CB, BMCs, and Bu showed sustained deletion of $V\beta11^+$ and $V\beta5.1/2^+CD4^+$ T cells first evident at day 28 after transplant (*C–F*). Recipients treated with CB and BMCs (no Bu) showed transient deletion of $V\beta11^+CD4^+$ T cells on day 28, but had $V\beta11^+$ and $V\beta5.1/2^+$ levels consistent with wild-type B6 levels (4–5% and 2–3%, respectively) after day 60 (*A*). Other recipients did not show any deletion of $V\beta11^+$ and $V\beta5.1/2^+CD4^+$ T cells. $V\beta8.1/2^+CD4^+$ T cells were not deleted by any group (data not shown). Similar results have been observed in 100 mice from multiple experiments. Data points represent the mean and SD for each group ($n = 3$).

secondary skin graft discussed above, there are several other possible reasons for this apparent discrepancy. The strain combination in the current study (B6 recipients and BALB/c donors) has been demonstrated to be vigorous in its ability to overcome the effect of CB, and thus provides a more challenging barrier for the induction of tolerance (34, 35). Second, in the current study, the histological examination of the heart grafts was performed at 300 days after transplant (100 days after rechallenge with a donor skin graft). This is a considerably longer follow-up than in the earlier reports. Third, there are several subtle differences in the treatment regimens between studies, such as the source and timing of blockade or donor cell administration relative to transplantation of the heart graft, that may have contributed to the different outcomes.

Recently, Russell et al. (4) have reported that successful chimerism induction strategies may, in certain circumstances, induce tolerance, but not prevent chronic rejection. In their studies, mixed chimerism was induced in B6 recipients from B10.A donors using a regimen consisting of total body irradiation, depletion of CD4 and CD8 cells and a single dose anti-mouse CD40L, followed by confirmation of tolerance by a challenge of donor-specific skin graft. Afterward, heart allografts were transplanted into the recipients with no further immunotherapy. Chronic vascular rejection was observed in allografts transplanted to these chimeric recipients. There are several possibilities for different results between Russell's and ours. First, it is possible that immune responses to heart-specific polymorphic Ag(s) might play a role in the rejection observed in their experiments. In our experiments, the induction of mixed chimerism and transplantation of the heart were performed simultaneously under the cover of CB, while in Russell's experiments mixed chimerism was established at least 100 days before heart transplantation. Thus, it is possible that transplantation of the heart under the cover of CB might tolerize Ag-specific T cells to heart-specific polymorphic Ag(s) not expressed by the bone marrow, whereas in the Russell experiments delayed heart transplantation would expose the recipient to these putative Ags in the absence of any immunosuppression, perhaps contributing to the observed vasculopathy. Second, not only alloantigen-dependent, but also alloantigen-independent factors can cause chronic rejection (36). Previous reports have suggested that cold ischemia/reperfusion injury can provoke chronic organ dysfunction and vascular remodeling and that administration of CTLA4-Ig can prevent these lesions (37, 38). Thus, it is possible that in our experiments CB could protect the heart graft from not only T cell-mediated responses, but also inhibit ischemia/reperfusion injury via mechanisms that are not completely understood. Third, as suggested by Russell's group, NK cells might be involved in the pathogenesis of obliterative vasculopathy in cardiac allografts. Several studies have provided evidence that the CD40/CD154 and CD28/B7 pathways may play an important role in the activation of NK cells (39). However, the recent report that NK cells play an important role in cardiac allograft rejection in $CD28^{-/-}$ mice suggests that blockade of this pathway alone is insufficient to inhibit NK-induced rejection. Thus, the incorporation of agents to block both the CD40 and CD28 pathways in the peritransplant period in our studies may synergistically inhibit NK cell-mediated injury that contributes to chronic vasculopathy. Finally, the duration of CB in our studies was considerably longer than in most earlier studies. Given the surprisingly long period of time (~60 days) to promote complete deletion of donor-reactive T cells, it is possible that prolonged CB plays an important role by protecting the allograft until the deletion process is complete.

In summary, the data presented have shown that several regimens incorporating CD28 and CD40 blockade with donor cell infusions prolong cardiac allograft survival and protect the allografts from peritransplant infiltration and immunologic injury. However, we have found that only a regimen consisting of CD28 and CD40 blockade together with a minimally myelosuppressive dose of Bu and donor BMCs promoted robust deletional tolerance. Importantly, this regimen also prevented the development of chronic

allograft vasculopathy. These data suggest that further development of regimens targeting the CD40 and CD28 pathways to promote chimerism and tolerance may have a significant impact on chronic rejection, an untreatable cause of clinical transplant failure.

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K. RELATED PROCEEDINGS APPENDIX

At the present time, there are no pending appeals or interferences related to this case.